IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Examiner: Basi, Nirmal Singh
	)	
Avi ASHKENAZI, *et al.*	)	Art Unit: 1646
	)	
Application Serial No. 09/909,088	)	Confirmation No: 1981
	)	
Filed: July 18, 2001	)	Attorney's Docket No. 39780-1618 P2C79
	)	
For: **SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING SAME**	)	**Customer No. 35489**

FILED VIA EFS
ON November 21, 2007

ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES
APPELLANTS' BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents -
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

On February 1, 2007, the Examiner made a Final rejection to pending Claims 39-47 and 49-52 and 55-58. A response and Notice of Appeal were filed on July 25, 2007.

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner. This Brief is timely filed requesting a two-month extension of time with fees.

The following constitutes Appellants' Brief on Appeal.

1. REAL PARTY IN INTEREST

The real party in interest is Genentech, Inc., South San Francisco, California, by an assignment of the parent application, U.S. Patent Application Serial No. 09/665,350 recorded July 9, 2001, at Reel 011964 and Frame 0181.

2. RELATED APPEALS AND INTERFERENCES

The claims pending in the current application are directed to a polypeptide referred to herein as "PRO335." There exists two related patent applications: 1) U.S. Patent Application Serial No. 09/903, 520, filed July 11, 2001 (containing claims directed to PRO335 polypeptides), and 2) U.S. Patent Application Serial No. 09/904,786, filed July 12, 2001 (containing claims directed to PRO335 antibodies). These applications are also under final rejection from the same Examiner and based upon the same type of outstanding rejections, and an appeal of these final rejections is being pursued independently and concurrently herewith.

3. STATUS OF CLAIMS

Claims 1-38, 48 and 53-54 were canceled without prejudice or disclaimer.

Claims 39-47, 49-52 and 55-58 stand rejected in this application and Appellants appeal the rejection of these claims.

A copy of the rejected claims involved in the present Appeal is provided in Section 8.

4. STATUS OF AMENDMENTS

No claim amendments were submitted after the final rejection. All previous amendments have been entered.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The invention claimed in the present application is related to isolated polynucleotides comprising a nucleic acid sequence encoding the polypeptide of SEQ ID NO:290 referred to in the present application as "PRO335," a nucleic acid sequence encoding the polypeptide of SEQ ID NO:290, or a nucleic acid sequence encoding the polypeptide of SEQ ID NO:290 lacking its associated signal peptide; or the full-length coding sequence of the nucleic acid sequence of SEQ ID NO:289; or a nucleic acid sequence of the full-length coding sequence of the cDNA deposited

under ATCC accession number 209927 (Independent Claim 44, and claims 45-47 and 49). The invention is further directed to nucleic acids having at least 80-99% sequence identity to nucleic acids encoding polypeptides of SEQ ID NO:290; or the nucleic acid sequence encoding the polypeptide of SEQ ID NO:290 lacking its associated signal peptide; or the full-length coding sequence of the nucleic acid sequence of SEQ ID NO:289; or the nucleic acid sequence of the cDNA deposited under ATCC accession number 209927, wherein the polypeptide encoded by said nucleic acid is an immunostimulant (Independent Claims 39-43).

The invention is further directed to vectors comprising these nucleic acids and host cells comprising such vectors (page 117 to page 123). PRO nucleic acid variants having at least about 80% nucleic acid sequence identity with a nucleic acid encoding for a full length PRO polypeptide sequence or a PRO polypeptide sequence lacking the signal peptide are described in the specification at page 55, line 2 to page 57, line 10, and for example, at page 69, line 25 to page 72, line 8.

The cDNA nucleic acid encoding PRO335 is described in the specification at, for example, page 184, line 21 to page 185, line 32 (Example 43), in Figure 101 and in SEQ ID NO:289. Page 63, lines 34-37 of the specification provides the description for Figures 101 and 102. The full-length PRO335 polypeptide having the amino acid sequence of SEQ ID NO:290 is described in the specification at, for example, page 50-51, lines 1-22, in Figure 102 and in SEQ ID NO:290.

Recombinant expression, characteristics and effects of the PRO335 polypeptides were disclosed in the specification, including in Examples 43, 54, 56, 74, and 77. The PRO335 polypeptides encoded by the claimed nucleic acids were shown to induce proliferation of stimulated T-lymphocytes in a mixed lymphocyte reaction as compared to controls (Example 74). PRO335 is also described as a polypeptide having homology to proteins of the leucine rich repeat superfamily, and particularly, are related to LIG-1 (page 30, line 11, to page 31, line 18, and page 110, lines 26-36). Example 74 (page 208) shows that PRO335 tested positive in the mixed lymphocyte reaction (MLR) assay, demonstrating that PRO335 is active as a stimulator of the proliferation of stimulated T-lymphocytes, and therefore would have utility in the treatment of conditions where the enhancement of an immune response would be beneficial. In addition,

Example 77 shows the ability of PRO335 to stimulate an immune response and induce inflammation at the site of injection in the skin vascular permeability assay, using the hairless guinea pig injected with the Evans blue dye as a model system.

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. Whether the data generated in the MLR assay (Example 74) satisfies the utility requirement under 35 U.S.C. § 101, and further, whether the data satisfies the enablement requirement set forth in 35 U.S.C. § 112, first paragraph for the invention claimed in Claims 39-47, 49-52 and 55-58.

II. Whether the data generated in the MLR assay (Example 74) satisfies the Enablement requirement set forth in 35 U.S.C. § 112, first paragraph, for the invention claimed in Claims 39-47, 49-52 and 55-58.

III. Whether the data generated in the MLR assay (Example 74) satisfies the Written Description requirement set forth in 35 U.S.C. § 112, first paragraph, for the invention claimed in Claims 39-47, 49-52 and 55-58.

7. ARGUMENT

Summary of the Arguments:

Issue I: Utility/ Enablement

Appellants submit that patentable utility for the PRO335 polypeptide is based upon data derived from the mixed leukocyte reaction (MLR) assay. The MLR assay is a well-established and accepted assay in the art for evaluating test compounds for their ability to stimulate T-lymphocyte proliferation *in vitro*. Example 74 of the instant specification shows that PRO335 tested positive in the mixed lymphocyte reaction (MLR) assay, demonstrating that PRO335 is active as a stimulator of the proliferation of stimulated T-lymphocytes, and therefore has utility in the treatment of conditions where the enhancement of an immune response would be beneficial, like to treat tumor progression/ regression in cancer. In fact, the Examiner himself acknowledges that MLR is an art accepted assay for identifying immunomodulatory compounds at least on page 3 and on page 11, paragraph 1 of the Final Office Action mailed February 1,

2007. Appellants have submitted several references throughout the prosecution of this case that supports the Appellants' position that the *in vitro* MLR assay **has been** successfully used to identify compounds having immunomodulatory activity *in vivo*, which the Examiner has acknowledged. By the priority date of the present application (September 17, 1998), it was well known that stimulators of T-cell proliferation would have utility in fighting diseases like: viral infections, including retroviral infections, (HIV infection or Epstein-Barr infection), or in the treatment of invasive cancers such as melanoma. Based on the utility for the PRO335 polypeptide, the nucleic acids encoding the PRO335 polypeptides are also claimed to have utility herein.

Appellants also note that the claimed variant nucleic acids having at least 80% nucleic acid sequence identity to the nucleic acid encoding the polypeptide sequence of SEQ ID NO:290 or the nucleic acid sequence of SEQ ID NO:289 and further, which recite the functional recitation "wherein said polypeptide is an immunostimulant." Thus, each claimed variant shares an immunostimulant property besides having sequence identity to the nucleic acid sequence encoding the PRO335 polypeptide. The specification provides ample guidance to the skilled artisan to identify variant nucleic acids with sequence identity and includes a detailed protocol of the MLR assay.

In addition, Appellants submitted with their Preliminary Amendment of August 30, 2004, a Declaration by Dr. Sherman Fong, who is an unquestionable expert in the field. The Declaration provides several examples of important clinical applications for immune stimulants which have shown activity in a mixed lymphocyte reaction assay, such as the chemokine IL-12, which finds utility in the treatment of melanoma due to its ability to stimulate immune response. Dr. Fong's declaration states that "a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity of at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant." The specification discloses that PRO335 has an activity of at least 180% of the control (see Example 74 of the instant specification).

Despite Dr. Fong's unequivocal statement, and the Examiner's acknowledgement that "the MLR assay is an accepted *in vitro* model for screening immunosuppressive agents for use in the prevention of graft-versus-host disease and graft rejection" (see page 15, second paragraph of the Final Office action of February 1, 2007), and the teachings of the present specification, the Examiner has asserted that "this biological activity does not correlate to use of the claimed nucleic acid in a therapeutically effective manner, as the asserted use of the claimed invention proposes." The Examiner adds that "the specification does not provide any values or data for the proteins tested in the assay. The specification does not provide any statistics for the values measured in the assay." The Examiner also takes issue with Dr. Fong's declaration and does not find it persuasive allegedly because "the expert has interest in the outcome of the case since (he) is listed as an inventor and is employed by the assignee." The Examiner questions the significance of the expert's conclusions based on alleged lack of use of proper controls. The Examiner erroneously concludes that undue experimentation would be need to practice the invention.

Appellants respectfully submit that, despite the Examiner's acknowledgment of PRO335 as an immunosuppressive molecule based on a positive MLR assay, the Examiner's general concern here seems to be with a requirement for statistical results, demonstration of controls, and the underlying therapeutic mechanism for its effectiveness, and not with the positive result itself. Appellants respectfully submit that the Examiner's concern is not a proper basis for a utility rejection. The Examiner seems to apply a standard that might be appropriate if the issue at hand were the **regulatory approval of a drug** based on the immunoenhancer activity of PRO335, but is **fully inappropriate for determining whether the "utility" standard of the Patent Statute is met**. The FDA, in reviewing an application for a new immunoenhancer drug, will indeed ask for actual numerical data, statistical analysis, therapeutic effectiveness and other specific information, before the drug is approved. However, the Patent and Trademark Office is not the FDA, and the standards of patentability are not the same as the standards for market approval.

Moreover, the evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration. Thus, to overcome the presumption of truth that an assertion of utility by the Appellant enjoys, the

Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner has made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the Appellant.

The Examiner has not shown that a lack of correlation typically exists between the results of the MLR assay in this instance and use of an immunomodulator such as PRO335 in a therapeutically effective manner. In fact, Appellants submit that the teachings within Picotti *et al.*, Campo *et al.* and Nishioka *et al.* discussed during prosecution, support the Appellants' position that the *in vitro* MLR assay **can** and **has been** successfully used to identify compounds having immunomodulatory activity *in vivo.* Appellants add that the Examiner has misinterpreted the specification, which clearly states what controls were used in the instant case, as will be elaborated upon below. In fact, based on such teachings, one of ordinary skill in the art would find it 'more likely than not' for molecules testing positive in the disclosed MLR assay, like PRO335, to have real-world therapeutic utility as immunostimulants *in vivo;* and would also know exactly how to use the claimed nucleic acids that encode PRO335 polypeptides, to treat disease conditions that are well- known and studied in the art: for example, in the treatment of viral infections and cancer, without any undue experimentation. Thus the Patent Office has failed to meet its initial burden of proof that Appellants' claims of utility are not substantial or credible.

Therefore, Appellants submit that when the proper legal standard is applied, one should reach the conclusion that the present application discloses at least one patentable utility for the claimed nucleic acids that encode PRO335 polypeptides that are useful since PRO335 polypeptides is useful as an immunostimulant. In view of the teachings of the instant specification and the knowledge in the art, one of ordinary skill in the art would understand exactly how to use the recited nucleic acids to treat a variety of diseases like viral infections, or cancer, diseases where immunostimulation is known to be therapeutic, without any undue experimentation.

Issue II: Enablement

Regarding the enablement rejection, Appellants note that the specification provides ample guidance to allow the skilled artisan to make and use those variant nucleic acids that

encode for PRO335 polypeptides that are useful in the treatment of conditions like viral infections or cancer, and further, one skilled in the art would know how to use these nucleic acids without any undue experimentation.

Accordingly, Appellants submit that the instant specification and the MLR assay suffices to provide enablement for the claimed subject matter,, without any undue experimentation.

Issue III: Written Description

Regarding the written description rejection, Appellants note that the specification provides ample guidance to allow the skilled artisan to identify those nucleic acids with 80-99% identity to the nucleic acid defined in SEQ ID NO.: 289. Further, the Applicants have provided a well-accepted *in vitro* MLR assay **can** and **has been** successfully used to identify compounds having immunomodulatory activity *in vivo* (Example 74). Moreover, the instant invention evidences the actual reduction to practice of full-length nucleic acid encoding PRO335 of SEQ ID NO:289, with or without its signal sequence, or encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209927. Therefore, the claimed nucleic acids are defined both by functional as well as structural features and would not encompass combinations much larger that 10^{23} and 10^{56} as the Examiner contends. Therefore, the Examiner's contention is not appropriate. Accordingly, Appellants submit that the instant specification and the MLR assay meet the written description standards set by the U.S.P.T.O. for the claimed subject matter.

These arguments are all discussed in further detail below under the appropriate headings.

Detailed Arguments:

ISSUE I: The Data Generated in the MLR Assay Satisfies the Utility/ Enablement Requirement of 35 U.S.C. §§101/112, First Paragraph for Claims 39-47, 49-52 and 55-58

Appellants submit that the results of the MLR assay in the instant specification (and in the priority U.S. Provisional Patent Application Serial No. 60/100,858) provides at least one credible, substantial and specific asserted utility for the claimed nucleic acids encoding PRO335 polypeptides under 35 U.S.C. §101/§112, first paragraph.

A. Legal Standard for Utility

According to 35 U.S.C. §101:

> Whoever invents or discovers any new and *useful* process, machine, manufacture, or composition of matter, or any new and *useful* improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title. (Emphasis added).

According to the Utility Examination Guidelines ("Utility Guidelines"), 66 Fed. Reg. 1092 (2001), an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility." Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that is to be diagnosed.

The requirement of "substantial utility" defines a "real world" use, and derives from the Supreme Court's holding in *Brenner v. Manson*, 383 U.S. 519, 534 (1966) stating that "The basic *quid pro quo* contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility." In. explaining the "substantial utility" standard, M.P.E.P. §2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, **any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient**, at least with regard to defining a "substantial" utility." (M.P.E.P. §2107.01, Emphasis added.) Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement, set forth in M.P.E.P. §2107 II(B)(1) gives the following instruction to patent examiners: "If the applicant has asserted that the claimed invention is useful for any particular practical purpose ... and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility."

Finally, the Utility Guidelines restate the Patent Office's long established position that any asserted utility has to be "credible." "Credibility is assessed from the perspective of one of

ordinary skill in the art in view of the disclosure and any other evidence of record ... that is probative of the applicant's assertions." (M.P.E.P. §2107 II(B)(1)(ii)). Such standard is presumptively satisfied unless the logic underlying the assertion is seriously flawed, or if the facts upon which the assertion is based are inconsistent with the logic underlying the assertion (Revised Interim Utility Guidelines Training Materials, 1999).

The case law has clearly established that applicants' statements of utility are usually sufficient, unless such statement of utility is unbelievable on its face.[1] The PTO has the initial burden to prove that applicant's claims of usefulness are not believable on their face.[2] In general, an applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. §101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope."[3, 4]

The well established case law is clearly reflected in the Utility Examination Guidelines ("Utility Guidelines"),[5] which acknowledge that an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility." Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that are to be diagnosed.

In explaining the "substantial utility" standard, M.P.E.P. §2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services

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[1] *In re Gazave*, 379 F.2d 973, 154 U.S.P.Q. (BNA) 92 (C.C.P.A. 1967).

[2] *Ibid.*

[3] *In re Langer*, 503 F.2d 1380,1391, 183 U.S.P.Q. (BNA) 288, 297 (C.C.P.A. 1974).

[4] *See also In re Jolles*, 628 F.2d 1322, 206 USPQ 885 (C.C.P.A. 1980); *In re Irons*, 340 F.2d 974, 144 USPQ 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 USPQ 209, 212-13 (C.C.P.A. 1977).

[5] 66 Fed. Reg. 1092 (2001).

based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility.'"[6] Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement,[7] gives the following instruction to patent examiners: "If the applicant has asserted that the claimed invention is useful for any particular practical purpose . . . and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility."

Appellants note that the phrase cited above "useful for any practical purpose" merely requires that an invention be useful, and does not require that it be *better* than other competing subject matter: "The Federal Circuit stated that a finding that "an invention that is an 'improvement' is not a prerequisite to patentability" since it "is possible for an invention to be less effective than existing devices but nevertheless meet the statutory criteria for patentability." (*Custom Accessories, Inc. v. Jeffrey-Allan Industries, Inc.)*[8]

In interpreting the utility requirement, in *Brenner v. Manson,*[9] the Supreme Court held that the *quid pro quo* contemplated by the U.S. Constitution between the public interest and the interest of the inventors required that a patent applicant disclose a "substantial utility" for his or her invention, *i.e.,* a utility "where specific benefit exists in currently available form."[10] The Court concluded that "a patent is not a hunting license. It is not a reward for the search, but

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[6] M.P.E.P. §2107.01.

[7] M.P.E.P. §2107 II(B)(1).

[8] *Custom Accessories, Inc. v. Jeffrey-Allan Industries, Inc.*, 807 F.2d 955, 1 USPQ2d 1196 (Fed. Cir. 1986).

[9] *Brenner v. Manson,* 383 U.S. 519, 148 U.S.P.Q. (BNA) 689 (1966).

[10] *Id.* at 534, 148 U.S.P.Q. (BNA) at 695.

compensation for its successful conclusion. A patent system must be related to the world of commerce rather than the realm of philosophy."[11]

Later, in *Nelson v. Bowler,*[12] the C.C.P.A. acknowledged that tests evidencing pharmacological activity of a compound may establish practical utility, even though they may not establish a specific therapeutic use. The Court held that "since it is crucial to provide researchers with an incentive to disclose pharmaceutical activities in as many compounds as possible, we conclude adequate proof of any such activity constitutes a showing of practical utility."[13]

Moreover, in *Cross v. Iizuka,*[14] the C.A.F.C. reaffirmed *Nelson*, and added that *in vitro* results might be sufficient to support practical utility, explaining that "*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results, *i.e.,* there is a reasonable correlation there between."[15] The Court perceived, perceived "No insurmountable difficulty" in finding that, under appropriate circumstances, "*in vitro* testing, may establish a practical utility."[16]

Furthermore, M.P.E.P. §2107.03 (III) states that:

> If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process.

Thus, the legal standard accepts that *in vitro* or animal model data is acceptable utility as long as the data is "reasonably correlated" to the pharmacological utility described.

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[11] *Id.* at 536, 148 U.S.P.Q. (BNA) at 696.

[12] *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (C.C.P.A. 1980).

[13] *Id.* at 856, 206 U.S.P.Q. (BNA) at 883.

[14] *Cross v. Iizuka*, 753 F.2d 1047, 224 U.S.P.Q. (BNA) 739 (Fed. Cir. 1985).

[15] *Id.* at 1050, 224 U.S.P.Q. (BNA) at 747.

[16] *Id.*

Compliance with 35 U.S.C. §101 is a question of fact.[17] The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration.[18] Accordingly, Appellants submit that in order to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that **it is more likely than not** that one of ordinary skill in the art would doubt the truth of the statement of utility. With respect to asserted therapeutic utilities based upon *in vitro* data, an applicant "does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty."[19] The law requires only that one skilled in the art should accept that such a correlation is **more likely than not to exist**. Appellants respectfully submit that when the proper evidentiary standard is applied, a correlation must be acknowledged.

Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence.

B. Proper Application of the Legal Standard

Appellants submit that the results of the mixed lymphocyte reaction (MLR) assay described in Example 74 of the instant specification (and in the priority U.S. Provisional Patent Application Serial No. 60/100,858) provides at least one credible, substantial and specific asserted utility for the claimed nucleic acids encoding PRO335 polypeptides under 35 U.S.C. §§101/112, first paragraph, based on the positive results for the PRO335 polypeptide in the MLR

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[17] *Raytheon v. Roper*, 724 F.2d 951, 956, 220 U.S.P.Q. (BNA) 592, 596 (Fed. Cir. 1983) *cert. denied,* 469 US 835 (1984).

[18] *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d (BNA) 1443, 1444 (Fed. Cir. 1992).

[19] M.P.E.P. §2107.03.

assay described at page 208 of the specification. Example 74 demonstrates that PRO335 is active as a stimulator of the proliferation of stimulated T-lymphocytes.

The MLR was a well-established assay at the priority date of the present application (September 17, 1998) for evaluating test compounds, such as the PRO335 polypeptide, for their ability to stimulate T-lymphocyte proliferation *in vitro*, and consequently, for assessing the immune response of an individual. The MLR assay is well-described in standard textbooks, including, for example, *Current Protocols in Immunology*, unit 3.12; edited by J E Coligan, A M Kruisbeek, D H Marglies, E M Shevach, W Strober, National Institutes of Health, Published by John Wiley & Sons, Inc. (1991) which is referenced in Example 74, the entire content of which is expressly incorporated by reference into the disclosure of the present application (see page 147, line 16-17). In brief, in this method, an immune response results upon mixing T-cells from antigenically distinct individuals under cell culture conditions. An MLR reaction can be monitored quantitatively by, for example, following the incorporation of tritiated thymidine during DNA synthesis, or by observing blast formation, or by other methods well known in the art.

According to the specification, positive increases over control in this assay are considered to be positive results, with increases of greater than or equal to 180% being preferred. However, any value greater than control indicates a stimulatory effect for the test protein. PRO335 (SEQ ID NO: 290) tested positive in this assay, using the described criteria. Example 74 further explains that compounds which stimulate proliferation of lymphocytes in this assay "are useful therapeutically where enhancement of an immune response is beneficial." Accordingly, PRO335 has utility in the treatment of conditions where the stimulation of lymphocyte proliferation would be desirable, like to treat tumor progression/ regression in cancer.

In further support of utility based upon the MLR assay, Appellants have submitted (with their Preliminary Amendment filed August 30, 2004) a Declaration by Sherman Fong, Ph.D. Dr. Fong is an inventor of the above-identified patent application, and an experienced scientist familiar with the MLR assay, which was used by him and others under his supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project, including PRO335.

The Fong Declaration explains how the MLR reaction was performed in the instant application using peripheral blood mononuclear cells (PBMCs), which contain responder T-cells, and allogenic, pre-treated (irradiated) PBMCs, which predominantly contained dendritic cells. Dr. Fong proceeds to explain (paragraph 7 of the Declaration) that dendritic cells are potent antigen-presenting cells that are able to "prime native T cells *in vivo*." Once activated by dendritic cells, the T-cells are capable of interacting with other antigen-presenting cells (B cells and macrophages) to produce additional immune responses from these cells.

As Dr. Fong states, "the MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously" (Paragraph 8 of the Fong Declaration). Dr. Fong also emphasizes that, immunostimulants are important and highly desirable in the treatment of cancer and in enhancing the effectiveness of previously identified treatments for cancer. Supportive evidence for this teaching comes from the art such as Steinman *et al.* (submitted as Exhibit B with the Amendment filed August 30, 2004) who state that "...**medicine needs therapies that enhance immunity or resistance to infections and tumors**" (page 1, column 1, line 7; emphasis added).

In paragraph 9 of his Declaration, Dr. Fong provides examples of important clinical applications for immune stimulants which have been shown to stimulate T-cell proliferation in the MLR assay. As Dr. Fong explains,

> "IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay [Gubler *et al.* PNAS 88, 4143 (1991) (Exhibit C)]. IL-12 was first identified in just such an MLR. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson *et al.* Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment

provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Thurner *et al.* J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)]."

Therefore, the art, as exemplified by Gubler *et al.* and Thurner *et al.*, in fact supports the Appellants' position that an MLR result is useful for identifying compounds with immunomodulatory activity *in vivo*. Dr. Fong concludes that (paragraph 10):

> It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant."

Accordingly, the positive results obtained in this assay clearly establish the immunostimulant utility for the PRO335 polypeptides and therefore, for the nucleic acids encoding PRO335 claimed in the present application, and the specification, in turn, enables one skilled in the art to use the compounds for the asserted purpose.

C. A *prima facie* case of lack of utility has not been established

As a preliminary matter, Appellants submit that, the evidentiary standard to be used throughout *ex parte* examination of a patent application is a preponderance of the totality of the evidence under consideration. Accordingly, Appellants submit that in order to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that **it is more likely than not** that one of ordinary skill in the art would doubt the truth of the statement of utility.

With respect to asserted **therapeutic utilities** based upon *in vitro* data, an applicant "does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty" (M.P.E.P. 2107.03). The law requires only that one skilled in the art should accept that such a correlation is **more likely than not to exist**. Appellants respectfully submit that when the proper evidentiary standard is applied, a correlation must be acknowledged.

Appellants have asserted, and the Examiner himself has acknowledged that the instant MLR assay is useful to identify compounds having immuno-modulatory activity *in vivo*. Accordingly, PRO335 would be useful to enhance immunostimulation in certain disease conditions such as cancer. Based on the well-published literature in the art, one skilled in the art would accept this assertion from the Applicant as credible and substantial.

Yet, the Examiner adds that the specification does not provide any values or data for the proteins tested in the assay, nor any statistical values measured in the assay.

Appellants respectfully submit that, the Examiner's general concern here seems to be with a requirement for statistical results, demonstration of controls, and the underlying therapeutic mechanism for its effectiveness, and not with the positive result itself. Appellants respectfully submit that the Examiner's concern is not a proper basis for a utility rejection. The Examiner seems to apply a standard that might be appropriate if the issue at hand were the **regulatory approval of a drug** based on the immunoenhancer activity of PRO335, but is **fully inappropriate for determining whether the "utility" standard of the Patent Statute is met**. The FDA, in reviewing an application for a new immunoenhancer drug, will indeed ask for actual numerical data, statistical analysis, therapeutic effectiveness and other specific information, before the drug is approved. However, the Patent and Trademark Office is not the FDA, and the standards of patentability are not the same as the standards for market approval. It is well established law that therapeutic utility sufficient under the patent laws is not to be confused with the requirements of the FDA with regard to safety and efficacy of drugs to be marketed in the United States. *Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994). Indeed, in *Nelson v. Bowler*, 626 F.2d 853, 856, 206 USPQ 881, 883 (CCPA 1980), the Federal Circuit found that the identification of a pharmacological activity of a compound provides an "immediate benefit to the public" and satisfies the utility requirement. This logically applies to utility for an immunostimulant as well. The identification of an immunostimulatory utility for a compound should suffice to establish an "immediate benefit to the public" and thus to establish patentable utility.

Furthermore, the mechanism of action need not be understood for attaining that utility. In fact, as stated by the Federal Circuit, "it is not a requirement of patentability that an inventor

correctly set forth, or even know, how or why the invention works." *In re Cortwright*, 165 F.2d 1353, 1359 (Fed. Cir. 1999). The Federal Circuit has also stated that "[a]n invention need not be the best or only way to accomplish a certain result, and it need only be useful to some extent and in certain applications: "[T]he fact that an invention has only limited utility and is not operable in certain applications is not grounds for finding lack of utility." *Envirotech Corp. v. Al George, Inc.* 730 F.2d 753,762, 221 USPQ 473,480 (Fed. Cir. 1984)." Thus, Appellants submit that such a concern is misplaced, and cannot properly form the basis of the rejections of the present claims.

Moreover, to overcome the presumption of truth that an assertion of utility by the Appellant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner has made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the Appellant.

The Examiner has not shown that a lack of correlation typically exists between the results of the MLR assay in this instance, and use of an immunomodulator such as PRO335 in a therapeutically effective manner. In fact, Appellants submit that the teachings within Picotti *et al.*, Campo *et al.* discussed during prosecution, support the Appellants' position that the *in vitro* MLR assay **can** and **has been** successfully used to identify compounds having immuno-modulatory activity *in vivo*.

D. The Fong Declaration supports "real world" utility for proteins that test positive in the MLR assay

The Examiner also takes issue with Dr. Fong's declaration and does not find it persuasive allegedly because "the expert has interest in the outcome of the case since (he) is listed as an inventor and is employed by the assignee." The Examiner questions the significance of the expert's conclusions based on alleged lack of use of proper controls. The Examiner erroneously concludes that undue experimentation would be need to practice the invention.

Appellants respectfully submit that Dr. Fong's statements are made under oath, and are asserted based on his vast knowledge and experience in the use and interpretation of the MLR assay. Furthermore, The case law has clearly established that in considering affidavit evidence,

the Examiner must consider all of the evidence of record anew.[20] "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument"[21] Furthermore, the Federal Court of Appeals held in *In re Alton*, "We are aware of no reason why opinion evidence relating to a fact issue should not be considered by an examiner"[22]. Appellants also respectfully draw the Examiner's attention to the Utility Examination Guidelines[23] which state, "Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered." The statement in question from an expert in the field (the Fong Declaration) states that "(i)t is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity of at least 180% of the control is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant." Therefore, barring evidence to the contrary regarding the above statement in the Fong Declaration, this rejection is improper under both the case law and the Utility guidelines.

The Examiner also mistakenly contends on page 16 of the Final Office Action (second paragraph) that "no 'particular antigen' is identified in the specification; there is no guidance as to how PRO335 could be used to boost the response to any antigen".

As Appellants have submitted previously, the PRO335 molecule, just like other immunostimulants (e.g., cytokines), stimulates cellular responses (cellular immunity) rather than humoral responses. Therefore, no "particular antigen" in the immune system need be identified. Therefore, this rejection is improper.

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[20] *In re Rinehart,* 531 F.2d 1084, 189 U.S.P.Q. 143 (C.C.P.A. 1976) and *In re Piasecki,* 745 F.2d. 1015, 226 U.S.P.Q. 881 (Fed. Cir. 1985).

[21] *In re Alton,* 37 U.S.P.Q.2d 1578 (Fed. Cir 1966) at 1584 quoting *In re Oetiker,* 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992)).

[22] *In re Alton, supra.*

[23] Part IIB, 66 Fed. Reg. 1098 (2001).

It was well known in the art at the time of filing of the instant application that T-cells are highly important in the body's natural defense mechanisms for fighting infections. For example, viral infections, such as HIV infection, were well known to result of a reduced T cell count. It was also well known at the time of filing that T cells could recognize tumor antigens and kill tumors. Therefore, one skilled in the art would reasonably know how to use the PRO335 immunostimulant, for instance, to boost the body's natural defense mechanisms for fighting infections or to recognize tumor antigens and/or to reduce and/or kill tumors.

The Examiner's misunderstanding of the instant MLR assay is further demonstrated in the Examiner's discussion of "controls" (see page 16 of the Final Office Action mailed February 1, 2007). The Examiner has asserted that "Current Protocols in Immunology" in fact describes many variables that must be controlled for. The Examiner alleges that in the instant application, no such controls such as for maximum response or for the inherent variability of individual responses are provided, no indication of the statistical significance of the result, no autologous controls, without giving any valid reason for doubting or questioning the assay controls used in the instant invention (emphasis added).

First of all, Appellants submit that these controls are only needed when the purpose of carrying out the MLR assay is to evaluate the properties of the stimulator cells. The comparisons to mismatched (maximum response) and autologous (background) controls allow one to determine the degree of HLA class II antigen similarity between the stimulator cells and the responder cells. Such determinations, however, are not relevant to the MLR assay of Example 74.

Applicants add that the MLR assay described in the instant specification is a comparative one (increases of greater than or equal to 180% is preferred), meaning that the utility is based upon a comparison of relative expression levels between a known polypeptide and an unknown PRO molecule. Additionally, Applicants expressly assert that the observed difference for PRO335 is significant as discussed in Example 74 of the instant specification. Therefore, contrary to the Examiner's position, controls were discussed in the specification. Further, regarding the Examiner requiring certain types of controls in the assay, again, Appellants submit that the Examiner appears to have misinterpreted the intent of the controls in the MLR assay

throughout this rejection. For instance, the mixing of the stimulator and responder cells in the instant MLR is expected to lead to T cell proliferation even in the absence of any test protein. The point of the MLR assay is to measure the extent to which the test protein can **enhance** the expected proliferation of the stimulated T cells. Appellants submit that the controls mentioned by the Examiner are only needed when the purpose of carrying out the MLR assay is to evaluate the properties of the stimulator cells. On the other hand, the purpose of the instant MLR assay, as discussed above, is to characterize test proteins such as PRO335, not stimulator cells. So, the precise extent to which the stimulator cells stimulate the responder cells is not significant; instead, what matters is the degree to which the test protein increases this response. The extent to which the test protein increases the response of the T cells is measured by comparison to a negative control reaction, which uses either cell culture medium, or a non immunostimulant molecule, CD4-IgG, as a negative control. Because the response in the test reaction is compared to a negative control reaction, and because both reactions use the same stimulator and responder cells at the same time, additional controls to determine the precise properties of these cells are not required.

Therefore, Appellants maintain that the assay controls used in the instant invention were appropriate, as discussed in clear detail in the Fong Declaration and throughout prosecution, and thus, the data for PRO335 in Example 74 (MLR assay) would be considered as meaningful, by one skilled in the art.

Appellants add that references Gubler *et al.* and Peterson *et al.* were submitted, not to show MLR activities of PRO335 (which is novel), but to show that other investigators used similar MLR assays to the one described in the instant specification, to conclude that their molecules stimulate T-cell proliferation and can be useful to enhance immune response. Appellants maintain their position regarding references Gubler *et al.* and Peterson *et al.*

Accordingly, Appellants respectfully submit that the Examiner's comments fail to support a *prima facie* case of lack of utility.

The art in fact supports the Appellants' position that an MLR result is useful for identifying compounds with immunomodulatory activity in vivo

Appellants submit that the references Picotti *et al.* and Campo *et al.* study allograft rejections and immunosuppression of graft rejection using test compounds *in vitro*. These references, in combination with others cited by Appellants, demonstrate that the art as a whole recognizes that the mixed lymphocyte reaction (MLR) is in fact a widely used *in vitro* assay for identifying immunomodulatory compounds.

For example, Picotti *et al.* confirm that "IL-12 is also a key cytokine involved in promoting cell mediated immune responses in vivo" (page 1459, col. 1). Thus, the fact that a molecule such as IL-12, which is a known immunostimulant *in vivo,* does not accelerate graft rejection supports Appellants' argument that graft rejection is a specific pathway that does not necessarily reflect general immunoregulatory function. Picotti *et al.* too draw a similar conclusion, suggesting that "the magnitude of Th1-driven alloimmune response may not correlate directly to the severity of graft rejection," perhaps because Th2-driven immune responses are more relevant to graft rejection (page 1459, col. 2). Therefore, Picotti *et al.*, provides evidence that a molecule which does not show in vivo activity in a specific graft versus host interactive pathway may still have general immunomodulatory activity.

Campo *et al.* set out to look for an inhibitor of MHC *in vitro* which would have the fewest side effects *in vivo* (see Abstract). The authors note that high concentrations of zinc "impair **all** T cell and monocyte function" (page 20; emphasis added). The authors took this impairment as an indicator of toxicity, and therefore intentionally used concentrations of zinc below that at which all T-cell function was impaired, in order to identify a concentration range that would not result in toxic effects. However, that does not mean that Campo *et al.* found zinc to have no immuno-suppressive activity *in vivo*. In fact, the authors conclude, based upon their MLC results, that "zinc **could become an immunosuppressant in transplantation medicine** without toxic side effects" (page 21; emphasis added). Thus Campo *et al.* supports Appellants' position that those of skill in the art would interpret the results of MLC assays as having physiological relevance.

In fact, Appellants note that the authors stated that the MLR is an important method with a good predictive value. For example, Campo *et al.* teach that "the human mixed lymphocyte culture (MLC) is an important method to test donor-recipient compatibility in bone marrow transplantation. It could be shown that cytokine release, especially IFN-γ, **has a very good predictive value with regard to the transplantation outcome**, as cytokines play a major role in the generation of an alloreactive immune response and for the induction of graft rejection ***in vivo***.....Landolfo *et al.* inhibited T-cell reactivity by the addition of anti-IFN-γ **both *in vitro* and *in vivo***" (see page 18; emphasis added). Further, Picotti *et al.* showed that the IL-12Rβ1 subunit was critical for IL-12 driven enhanced alloimmune response ***in vitro* and *in vivo*** (see abstract). Thus, while there are instances of unpredictability using the MLR assay, there are many studies showing predictable results, including studies from Picotti, Landolfo and the IFN-γ study. Finally, Campo *et al.* teaches that "cyclosporin A, FK506, and other substances are used to prevent graft rejection. **In vitro experiments revealed an inhibition of the MLC**" (page 16). Thus the teachings of Campo *et al.* confirm that inhibition of the MLR is observed for known immunoinhibitory molecules, that are in actual clinical use.

Thus, in fact, references Picotti *et al.* and Campo *et al.* support the Appellants' position that it is more likely than not that the *in vitro* MLR assay can be successfully used to identify compounds having immunomodulatory activity *in vivo*.

E. One Skilled in the Art would know how to make and use the antibodies without undue experimentation based on the teachings in the art and in the specification

The fact remains that the results of the MLR assay were positive, indicating that PRO335 is an immunostimulant. The Examiner 's concern that the results require undue experimentation further, do not negate the positive results of the assay, that is PRO335 is a an immunostimulant molecule, and further, do not negate the assertion of utility for PRO335 antibodies. As discussed above, one of ordinary skill in the art in possession of these results would, more likely than not, acknowledge that the PRO335 polypeptides are useful as an immunostimulant agents and therefore, that antibodies to the PRO335 polypeptide for reducing the undesirable effects of immunostimulation in disease conditions (for example, tissue destruction). Moreover, as the

M.P.E.P. states, "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation."[24] As discussed above, a considerable amount of experimentation is permissible, if it is merely routine.

In view of the above, Appellants submit that a valid case for utility has been made and would be considered credible by a person of ordinary skill in the art. Indeed, the logic underlying Appellants' assertion that the PRO335 polypeptides would be useful as an immunostimulant or in providing antibodies for inhibiting immunostimulation is not inconsistent with the general knowledge in the art, and would be considered credible by a person skilled in the art. Further, Appellants respectfully submit that the Examiner's comments fail to support a *prima facie* case of lack of utility.

Accordingly, Appellants respectfully request reconsideration and reversal of the enablement rejection of Claims 39-43 under 35 U.S.C. §112, first paragraph.

ISSUE II: The Data Generated in the MLR Assay Satisfies the Enablement Requirement of 35 U.S.C. § 112, First Paragraph for Claims 39-47, 49-52 and 55-58

In this regard, Appellants refer to the arguments and information presented above in response to the issue of utility, and those arguments are incorporated by reference herein. Appellants submit that, as discussed above, the MLR assay demonstrates utility for the PRO335 polypeptide for the treatment of conditions where the stimulation of lymphocyte proliferation would be desirable, including viral infections such as HIV and Epstein-Barr, and cancers such as melanoma. The present claims recite nucleic acids encoding polypeptides that induce an inflammatory response. Support for this recitation is found in Example 74 which describes the MLR assay in which PRO335 polypeptides is an immunostimulant. Based on such a utility, one of skill in the art would know exactly how to use the claimed nucleic acids encoding PRO335 for the treatment of conditions where enhancement of an immune response is beneficial, without any undue experimentation.

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[24] M.P.E.P. §2164.01 citing *In re Certain Limited-charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd sub nom. Massachusetts Institute of Technology v A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985).

Appellants also note that the claimed variants all share the functional recitation: "wherein said polypeptide is an immunostimulant." Example 74 of the present application provides detailed protocols for the MLR assay, including the extensive step-by-step guidance from Current Protocols in Immunology, which is explicitly incorporated into the specification by reference. By following the disclosure in the specification, one skilled in the art can easily test whether a variant PRO335 polypeptide encoded by the claimed nucleic acids is capable of stimulating proliferation of T-lymphocytes. Appellants claim only those nucleic acids that encode for proteins which meet both recitations of the claims, structural and functional. So, the breadth of the claims are clearly defined by both the structural and functional recitations.

Further, the specification provides detailed step-by-step guidance as to how to identify and make variant nucleic acids encoding the PRO335 polypeptides. The specification also provides ample guidance to allow the skilled artisan to make and identify those polypeptides which meet the limitations of the claims. Therefore, one of skill in the art could readily test a polypeptide encoded by the claimed nucleic acids to determine whether it is capable of inducing inflammation by the methods set forth in Example 74. As the M.P.E.P. states, "The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-charge cell Culture Microcarriers, 221 USPQ 1165, 1174* (Int'l Trade Comm'n 1983), *aff'. sub nom., Massachusetts Institute of Technology v A.B. Fortia,* 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985) M.P.E.P. §2164.01. A considerable amount of experimentation is permissible, if it is merely routine.

Therefore, Appellants respectfully submit that the specification provides ample guidance such that one of skill in the art could readily test a variant nucleic acid encoding a polypeptide of PRO335 to determine whether the polypeptide is capable of stimulating proliferation of T-lymphocytes by the methods set forth in Example 74. This biological activity together with the well defined relatively high degree of sequence identity and general knowledge in the art at the time the invention was made, sufficiently defines the claimed genus such that, one skilled in the art, at the effective date of the present application, without undue experimentation.

Accordingly, Appellants believe the rejections of Claims 39-47, 49-52 and 55-58 under 35 U.S.C. §112, first paragraph, to be improper, and respectfully request withdrawal of these rejections.

ISSUE III: The Data Generated in the MLR Assay Satisfies the Written Description Requirement of 35 U.S.C. § 112, First Paragraph for Claims 39-43, 52 and 55-58

Claims 39-43, 52 and 55-58 stand rejected under 35 U.S.C. §112, first paragraph as allegedly containing "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." In particular, the Examiner has asserted that "Apart from the polynucleotide of SEQ ID NO: 289 encoding the polypeptide of 290, the particular conserved structures or other distinguishing structural features critical for a specific activity of PRO335 are not disclosed. Thus, the claims are drawn to genus of polynucleotides that is deined only by sequence identity and general activity (applicable to many other polynucleotides) and no specific activity that can be associated with any specific domains of PRO335." (Pages 17-18 of the Final Office Action mailed February 1, 2007).

Appellants respectfully disagree.

A. The Legal Test for Written Description

The well-established test for sufficiency of support under the written description requirement of 35 U.S.C. §112, first paragraph is "whether the disclosure of the application as originally filed reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter, rather than the presence or absence of literal support in the specification for the claim language."[25] [26] The adequacy of written description support is a

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[25] *In re Kaslow*, 707 F.2d 1366, 1374, 212 USPQ 1089, 1096 (Fed. Cir. 1983).

[26] *See also Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116 (Fed. Cir. 1991).

factual issue and is to be determined on a case-by-case basis.[27] The factual determination in a written description analysis depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure.[28, 29]

In *Environmental Designs, Ltd. v. Union Oil Co.*,[30], the Federal Circuit held, "Factors that may be considered in determining level of ordinary skill in the art include (1) the educational level of the inventor; (2) type of problems encountered in the art; (3) prior art solutions to those problems; (4) rapidity with which innovations are made; (5) sophistication of the technology; and (6) educational level of active workers in the field."[31] Further, the "hypothetical 'person having ordinary skill in the art' to which the claimed subject matter pertains would, of necessity have the capability of understanding the scientific and engineering principles applicable to the pertinent art."[32, 33]

B. The Disclosure Provides Sufficient Written Description for the Claimed Invention

Appellants respectfully submit that the instant specification evidences the actual reduction to practice of the nucleic acid of SEQ ID NO: 289. Appellants also note that the claimed nucleic acid variants all share the functional recitation: "wherein said polypeptide is an immunostimulant." Example 74 of the present application provides detailed protocols for the

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[27] *See e.g., Vas-Cath*, 935 F.2d at 1563; 19 USPQ2d at 1116.

[28] *Union Oil v. Atlantic Richfield Co.*, 208 F.2d 989, 996 (Fed. Cir. 2000).

[29] *See also* M.P.E.P. §2163 1l(A).

[30] 713 F.2d 693, 696, 218 USPQ 865, 868 (Fed. Cir. 1983), *cert. denied*, 464 U.S. 1043 (1984).

[31] *See also* M.P.E.P. §2141.03.

[32] *Ex parte Hiyamizu*, 10 USPQ2d 1393, 1394 (Bd. Pat. App. & Inter. 1988) (emphasis added).

[33] S*ee also* M.P.E.P. §2141.03.

MLR assay, including the extensive step-by-step guidance from Current Protocols in Immunology, which is explicitly incorporated into the specification by reference. The disclosure shows how to test whether a variant PRO335 polypeptide encoded by the claimed nucleic acids is capable of stimulating proliferation of T-lymphocytes. So the specification provides ample guidance to make and identify those polypeptides encoded by the claimed nucleic acids which meet the limitations of the claims. Appellants claim only those nucleic acids that encode for proteins which meet both recitations of the claims, structural and functional.

In this regard, the Examiner's contention that the number of possible combinations of claimed nucleic acids is much larger that 10^{23} and 10^{56} is absurd and has no evidentiary basis. The breadth of the claims are clearly defined by both, structural and functional recitations, which are clearly defined in the specification. The specification provides detailed step-by-step guidance as to how to identify and make variant nucleic acids encoding the PRO335 polypeptides. PRO nucleic acid variants having at least about 80% nucleic acid sequence identity with a nucleic acid encoding for a full length PRO polypeptide sequence or a PRO polypeptide sequence lacking the signal peptide are described in the specification at page 55, line 2 to page 57, line 10, and for example, at page 69, line 25 to page 72, line 8. The functional MLR assay is clearly disclosed in the instant specification at Example 74, and extensive step-by-step guidance from Current Protocols in Immunology is explicitly incorporated into the specification, by reference. Thus, the MLR biological activity, together with the well defined high degree of sequence identity, and general knowledge in the art at the effective date of the present application, sufficiently defines the claimed genus such that one skilled in the art would know that the Appellants possessed the claimed polynucleotide variants.

Accordingly, Appellants believe the rejections of Claims 39-43, 52 and 55-58 under 35 U.S.C. §112, first paragraph, to be improper, and respectfully request withdrawal of these rejections.

CONCLUSION

For the reasons given above, Appellants submit that the MLR assay disclosed in Example 74 of the specification provides at least one patentable utility for the PRO335 polypeptides of Claims 39-47, 49-52 and 55-58. In addition, Claims 39-47, 49-52 and 55-58 meet the requirements of 35 U.S.C. §112, first paragraph - enablement and written description. Accordingly, reversal of all the rejections of Claims 39-47, 49-52 and 55-58 is respectfully requested.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. **08-1641** (referencing Attorney's Docket No. **39780-1618 P2C79**).

Respectfully submitted,

Date: November 21, 2007

By:_____
Panpan Gao (Reg. No. 43,626)
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SV2318021 v1

8. CLAIMS APPENDIX

Claims on Appeal

39. An isolated nucleic acid having at least 80% nucleic acid sequence identity to:

(a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);

(b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;

(c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);

(d) the nucleic acid sequence of (SEQ ID NO: 289);

(e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or

(f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927,

wherein the polypeptide encoded by said nucleic acid is an immunostimulant.

40. The isolated nucleic acid of Claim 39 having at least 85% nucleic acid sequence identity to:

(a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);

(b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;

(c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290):

(d) the nucleic acid sequence of (SEQ ID NO: 289);

(e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or

(f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927,

wherein the polypeptide encoded by said nucleic acid is an immunostimulant.

41. The isolated nucleic acid of Claim 39 having at least 90% nucleic acid sequence identity to:

(a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);

(b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;

(c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290):

(d) the nucleic acid sequence of (SEQ ID NO: 289);

(e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or

(f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927,

wherein the polypeptide encoded by said nucleic acid is an immunostimulant.

42. The isolated nucleic acid of Claim 39 having at least 95% nucleic acid sequence identity to:

(a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);

(b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;

(c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290):

(d) the nucleic acid sequence of (SEQ ID NO: 289);

(e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or

(f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927,

wherein the polypeptide encoded by said nucleic acid is an immunostimulant.

43. The isolated nucleic acid of Claim 39 having at least 99% nucleic acid sequence identity to:

(a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);

(b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;

(c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290):

(d) the nucleic acid sequence of (SEQ ID NO: 289);

(e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or

(f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927,

wherein the polypeptide encoded by said nucleic acid is an immunostimulant.

44. An isolated nucleic acid comprising:

(a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290);

(b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide;

(c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290);

(d) the nucleic acid sequence of (SEQ ID NO: 289);

(e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or

(f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

45. The isolated nucleic acid of Claim 44 comprising a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290).

46. The isolated nucleic acid of Claim 44 comprising a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290), lacking its associated signal peptide.

47. The isolated nucleic acid of Claim 44 comprising a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290).

49. The isolated nucleic acid of Claim 44 comprising the nucleic acid sequence of (SEQ ID NO: 289).

50. The isolated nucleic acid of Claim 44 comprising the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289).

51. The isolated nucleic acid of Claim 44 comprising the full-length coding sequence of the cDNA deposited under ATCC accession number 209927.

52. An isolated nucleic acid molecule consisting of a fragment of the nucleic acid sequence of SEQ ID NO: 289, or a complement thereof, that specifically hybridizes under stringent conditions to:

(a) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO: 290):

(b) a nucleic acid sequence encoding the polypeptide of (SEQ ID NO 290), lacking its associated signal peptide;

(c) a nucleic acid sequence encoding the extracellular domain of the polypeptide of (SEQ ID NO: 290):

(d) the nucleic acid sequence of (SEQ ID NO: 289);

(e) the full-length coding sequence of the nucleic acid sequence of (SEQ ID NO: 289); or

(f) the full-length coding sequence of the cDNA deposited under ATCC accession number 209927,

wherein said stringent conditions are hybridization in 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran

sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

55. A vector comprising the nucleic acid of Claim 39.

56. The vector of Claim 55, wherein said nucleic acid is operably linked to control sequences recognized by a host cell transformed with the vector.

57. A host cell comprising the vector of Claim 55.

58. The host cell of Claim 57, wherein said cell is a CHO cell, an *E. coli* or a yeast cell.

9. EVIDENCE APPENDIX

1. Declaration of Sherman Fong, Ph.D. under 35 C.F.R §1.132, with attached Exhibits A-E:

A. Current Protocols in Immunology, Vol. 1, Richard Coico, Series Ed., John Wiley & Sons, Inc., 1991, Unit 3.12.

B. Steinman, R.M., "The dendritic cell advantage: New focus for immune-based therapies," *Drug News Perspect.* **13**:581-586 (2000).

C. Gubler, U. *et al.,* "Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor," *Proc. Natl. Acad. Sci. USA* **88**:4143-4147 (1991).

D. Peterson, A.C. *et al.,* "Immunization with melan-A peptide-pulsed peripheral blood mononuclear cells plus recombinant human interleukin-12 induces clinical activity and T-cell responses in advanced melanoma," *J. Clin. Oncol.* **21**:2342-2348 (2003).

E. Thurner, B. *et al.,* "Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T-cells and induces regression of some metastases in advanced stage IV melanoma," *J. Exp. Med.* **190**:1669-1678 (1999).

2. Kahan, Barry D., "Immunosuppressive therapy," *Curr. Opin. Immunol.* **4**:553-560 (1992).

3. Picotti, J.R. et al., "Interleukin-12 (IL-12)-driven alloimmune responses in vitro and in vivo," *Transplantation* **67**:1453-1460 (1999).

4. Campo, C.A. et al., "Zinc inhibits the mixed lymphocyte culture," *Biological Trace Element Research*, **79**:15-22 (2001)

Item 1 was submitted with Preliminary Amendment filed August 30, 2004, and was noted as considered by the Examiner on November 17, 2004.

Items 2-4 were made of record by the Examiner in the Office Action mailed May 30, 2006.

10. RELATED PROCEEDINGS APPENDIX

None - no decision rendered by a Court or the Board in any related proceedings identified above.

APPENDIX 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Docket No.:

Serial No.: Group Art Unit:

Filing Date: Examiner:

For:

DECLARATION OF SHERMAN FONG, Ph.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Sherman Fong, Ph.D. declare and say as follows: -

1. I was awarded a Ph.D. in Microbiology by the University of California at Davis, CA in 1975.

2. After postdoctoral training and holding various research positions at Scripps Clinic and Research Foundation, La Jolla, CA, I joined Genentech, Inc., South San Francisco, CA in 1987. I am currently a Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc.

3. My scientific Curriculum Vitae is attached to and forms part of this Declaration.

4. I am familiar with the Mixed Lymphocyte Reaction (MLR) assay, which has been used by me and others under my supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project.

5. The MLR assay is a well known and widely used proliferative assay of T-cell function, the basic protocols of which are described, for example, in Current Protocols in Immunology Vol. 1, Richard Coico, Series Ed., JohnWiley & Sons, Inc., 1991, Unit 3.12. (Exhibit A). This publication is incorporated by reference in the description of the MLR protocol in the present application.

6. The T-lymphocytes or "T-cells" of our immune system can be induced to proliferate by a variety of agents. The MLR assay is designed to study a particularly important induction mechanism whereby responsive T-cells are cultured together (or "mixed"), with other lymphocytes that are "allogeneic", e.g. lymphocytes that are taken from different individuals of the same species. In the MLR protocol of the present application, a suspension of PBMCs that includes responder T-cells, is cultured with allogeneic PBMCs that predominantly contain dendritic cells. According to the protocol, the allogeneic "stimulator" PBMCs are irradiated at a dose of 3000 Rad. This irradiation is done in order to create a sample of cells that has mainly dendritic cells. It is known that the dendritic cell population among the PBMCs are differentially affected by irradiation. At low doses (500-1000 Rad), the proliferation of most cells, including the B cells in the PBMCs, is preserved, however, at doses above 2000 Rad, this function of B cells is abolished. Dendritic cells on the other hand, maintain their antigen presentation function even at a 3000 Rad dose of radiation. (See, e.g. Current Protocols in Immunology, *supra*, at 3.12.9). Accordingly, under the conditions of the MLR assay used to test the PRO polypeptides of the present invention, the stimulator PBMCs remaining after irradiation are essentially dendritic cells.

7. Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naive T cells *in vivo*. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells. For further details about the properties and role of dendritic cells in immune-based therapies see, e.g. Steinman, Drug News Perspect. 13(10):581-586 (Exhibit B).

8. The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

9. Such immune stimulants find important clinical applications. For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Thurner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)].

10. It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant. Some PRO polypeptides do the reverse, and give inhibition of T-cell proliferation in the MLR assay. It is my considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases.

Dated: 6/16/04

By: Sherman Fong

Sherman Fong, Ph.D.

2041362

Sherman Fong, Ph.D.

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Education:

1978 - 1980 Postdoctoral Fellow in Immunology, Scripps Clinic and Research Institute of Scripps Clinic, Research Foundation, La Jolla, California

1975 - 1978 Postdoctoral Fellow in Immunology, University of California at San Francisco, San Franciso, California

1970 - 1975 Ph.D. in Microbiology, University of California at Davis, California

1966 - 1970 B.A. in Biology/Microbiology, San Francisco State University, San Francisco, California

Professional Positions:
Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc. South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:
Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Resarch Grant, NIH (1985)

Recipient: P.I., Resarch Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Commitee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmcological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the **Biochemical Subteam for Protropin® Project** team.

Served as Immunologist on the **Met-less hGH and Dnase project teams**, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in1989-1990 on the **CD4-IgG project team** carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte β2 integrins. Provided preclinical scientific data to **Anti-CD18 project team**

supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, **Research Project Team leader** for small molecule $\alpha 4\beta 1$ integrin antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped established and responsible for maintaining current research collaboration with Roche-Nutley. Project transfered to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against $\alpha 4\beta 7$ intergin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn';s disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, **Research Project Team Biology Leader** (1996-present) for small molecule antagonists for $\alpha 4\beta 7$/MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as **Ad Hoc reveiwer** on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as Scientific advisor with staff of the **Business Development** Office on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001 Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The β2 Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process – Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process – Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin α4β7 Attentuates Inflammatory Bowel Diseases", in Sympoium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:
Dennis A. Carson, Sherman Fong, Pojen P. Chen.
U.S. Patent Number 5,068,177: Anti-idiotype Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.
U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent 5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.
U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colits, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen
U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood
U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams, William Wood . U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura
US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood
US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

Publications:

1. Scibienski R, Fong S, Benjamini E: Cross tolerance between serologically non-cross reacting forms of egg white lysozyme. J Exp Med 136:1308-1312, 1972.

2. Scibienski R, Harris M, Fong S, Benjamini E: Active and inactive states of immunological unresponsiveness. J Immunol 113:45-50, 1974.

3. Fong S: Studies on the relationship between the immune response and tumor growth. Ph D Thesis, 1975.

4. Benjamini E, Theilen G, Torten M, Fong S, Crow S, Henness AM: Tumor vaccines for immunotherapy of canine lymphosarcoma. Ann NY Acad Sci 277:305, 1976.

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10. Tsoukas CD, Carson DA, Fong S, Pasquali J-L, Vaughan JH: Cellular requirements for pokeweed mitogen induced autoantibody production in rheumatoid arthritis. J Immunol 125:1125-1129, 1980.

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12. Fong S, Pasquali J-L, Tsoukas CD, Vaughan JH, Carson DA: Age-related restriction of the light chain heterogeneity of anti-IgG antibodies induced by Epstein-Barr virus stimulation of human lymphocytes in vitro. Clin Immunol Immunopathol 18:344, 1981.

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15. Fong S, Tsoukas CD, Pasquali J-L, Fox RI, Rose JE, Raiklen D, Carson DA, Vaughan JH: Fractionation of human lymphocyte subpopulations on immunoglobulin coated petri dishes. J Immunol Methods 44:171-182, 1981.

16. Pasquali J-L, Tsoukas CD, Fong S, Carson DA, Vaughan JH: Effect of Levamisole on pokeweed mitogen stimulation of immunoglobulin production in vitro. Immunopharmacology 3:289-298, 1981.

17. Pasquali J-L, Fong S, Tsoukas CD, Hench PK, Vaughan JH, Carson DA: Selective lymphocyte deficiency in seronegative rheumatoid arthritis. Arthritis Rheum 24:770-773, 1981.

18. Fong S, Fox RI, Rose JE, Liu J, Tsoukas CD, Carson DA, Vaughan JH: Solid- phase selection of human T lymphocyte subpopulations using monoclonal antibodies. J Immunol Methods 46:153-163, 1981.

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20. Carson DA, Pasquali J-L, Tsoukas CD, Fong S, Slovin SF, Lawrance SK, Slaughter L, Vaughan JH: Physiology and pathology of rheumatoid factors. Springer Semin Immunopathol 4:161-179, 1981.

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22. Seybold M, Tsoukas CD, Lindstrom J, Fong S, Vaughan JH: Acetylcholine receptor antibody production during leukoplasmapheresis for Myasthenia Gravis. Arch Neurol 39:433-435, 1982.

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24. Sabharwal UK, Vaughan JH, Fong S, Bennett P, Carson DA, Curd JG: Activation of the classical pathway of complement by rheumatoid factors: Assessment by radioimmunoassay for C4. Arthritis Rheum 25:161-167, 1982.

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26. Fong S, Miller JJIII, Moore TL, Tsoukas CD, Vaughan JH, Carson DA: Frequencies of Epstein-Barr virus inducible IgM anti-IgG B lymphocytes in normal children and in children with Juvenile Rheumatoid Arthritis. Arthritis Rheum 25:959-965, 1982.

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29. Tsoukas CD, Carson DA, Fong S, Slovin SF, Fox RI, Vaughan JH: Lysis of autologous Epstein-Barr virus infected B cells by cytotoxic T lymphocytes of rheumatoid arthritis patients. Clin Immunol Immunopathol 24:8-14, 1982.

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34. Fong S, Vaughan JH, Carson DA: Two different rheumatoid-factor producing cell populations distinguished by the mouse erythrocyte receptor and responsiveness to polyclonal B cell activators. J Immunol 130:162-164, 1983.

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36. Fong S: Solid-phase panning for the fractionation of lymphoid cells. In: Cell separation: methods and selected applications. Pretlow TG, Pretlow TP (eds.) pp. 203-219. Academic Press, New York, 1983.

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41. Fong S, Gilbertson TA, Chen PP, Vaughan JH, Carson DA: Modulation of human rheumatoid factor-specific lymphocyte responses with a cross- reactive anti-idiotype bearing the internal image of antigen. J Immunol 132:1183-1189, 1984.

42. Chen PP, Houghten RA, Fong S, Rhodes GH, Gilbertson TA, Vaughan JH, Lerner RA, Carson DA: Anti-hypervariable region antibody induced by a defined peptide. A new approach for studying the structural correlates of idiotypes. Proc Natl Acad Sci USA 81:1784-1788, 1984.

43. Fox RI, Fong S, Tsoukas CD, Vaughan JH: Characterization of recirculating lymphocytes in rheumatoid arthritis patients: Selective deficiency of natural killer cells in thoracic duct lymph. J Immunol 132:2883-2887, 1984.

44. Chen PP, Fong S, Normansell D, Houghten RA, Karras JG, Vaughan JH, Carson DA: Delineation of a cross-reactive idiotype on human autoantibodies with antibody against a synthetic peptide. J Exp Med 159:1502-1511, 1984.

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47. Fong S, Chen PP, Vaughan JH, Carson DA: Origin and age-associated changes in the expression of a physiologic autoantibody. Gerontology 31:236-250, 1985.

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EXHIBIT A

CURRENT PROTOCOLS IN IMMUNOLOGY

VOLUME 1

John Wiley & Sons, Inc.

Library of Congress Cataloging in Publication Data:

Current protocols in immunology / edited by John E. Coligan ... [et al.].
p. cm.
Kept up to date by quarterly supplements.
4 vols. Includes index.
ISBN 0-471-52276-7
1. Immunology—Laboratory manuals I. Coligan, John E.
[DLM: 1. Clinical Protocols. 2. Immunologic Techniques. QW 525 C976]
QR183.C87 1991
616.07'0'072—dc20
DNLM/DLC
for Library of Congress 90-15726
CIP

Printed in the United States of America

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Proliferative Assays for T Cell Function

A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes—in contrast to helper function for B lymphocytes (*UNIT 3.10*) or cytotoxicity (*UNIT 3.11*)—proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. In addition, the assays described in this unit form the basis for identifying the appropriate cellular population that might be used to obtain T cell clones (*UNIT 3.13*) or T cell hybridomas (*UNIT 3.14*).

The assays have been divided into two groups on the basis of whether they are used to stimulate primed or unprimed T lymphocytes. The first basic protocol describes the use of agents that are capable of activating unprimed T lymphocytes in culture either by pharmacologic means (calcium ionophore and phorbol ester stimulation), by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3, anti-TCR-γδ, or anti-TCR-αβ monoclonal antibodies), by cross-linking the receptors on certain subpopulations of T cells with monoclonal antibodies specific for the *V* regions of β chains of the TCR (anti-Vβ) or with enterotoxins specific for certain *V*β-chain regions, or by indirectly cross-linking the TCR (lectins or monoclonal antibodies to non-TCR antigens). The first alternate protocol describes the use of plate-bound antibodies specific for the TCR to stimulate proliferation. The second alternate protocol describes the activation of unprimed T cells to cell-associated antigens in the mixed leukocyte reaction (MLR). The first support protocol describes the preparation and use of T cell–depleted accessory or stimulator cells and the second support protocol describes methods for blocking accessory cell proliferation. Finally, the second basic protocol describes the induction of a T cell proliferative response to soluble protein antigens or to cell-associated antigens against which the animal has been primed in vivo.

The assays in this unit employ murine T lymphocytes. Induction of proliferative responses of murine B lymphocytes is described in *UNIT 3.10*. Related assays for use with human peripheral blood lymphocytes are described in *UNIT 7.9*.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

ACTIVATION OF UNPRIMED T CELLS

BASIC PROTOCOL

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins. The commentary briefly describes the specificities of these agents, while Table 3.12.1 lists sources and concentrations for use in this protocol. Although this procedure is intended to measure proliferation of T cells specifically, in many cases induction of T cell proliferation is dependent on the presence of non–T cells that function as accessory cells. The latter provide additional costimulatory signals for T cell proliferation as well as cross-link (via their Fc receptors) monoclonal antibodies bound to cell-surface antigens. The requirement for non–T accessory cells varies with the nature of the stimulatory ligand and can range from absolute dependence to accessory cell–independent T cell activation (see Table 3.12.1). The activation is calculated after determining the difference in incorporation of [^{3}H]thymidine between stimulated and control cells.

Contributed by Ada M. Kruisbeek and Ethan Shevach
Current Protocols in Immunology (1991) 3.12.1-3.12.14

Table 3.12.1 Agents Used to Activate Unprimed T Cells in Proliferative Assays

Agent[a]	Source/ cat. no.[b]	Concentration	Accessory cells[c]	Mode of action, etc.
PMA	SIG P8139	1-10 ng/ml	No	Use with ionomycin or A23187; pharmacologic
Ionomycin	CAL 407950	200-500 ng/ml	No	Use with PMA; pharmacologic
A23187	CAL 100105	100-500 ng/ml	No	Use with PMA; pharmacologic
PHA	WD HA16	1-5 μg/ml	Yes	Indirect TCR cross-linking
Con A	PH 17-0450-01	1-10 μg/ml	Yes	Indirect TCR cross-linking
Anti-Thy-1	PG mAb-G7	1-50 μg/ml	Yes[c]	Indirect TCR cross-linking
Anti-CD3	PG HM-CD3	0.1-5 μg/ml	Yes[c]	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-αβ	PG HM-AB-TCR	0.1-10 μg/ml	Yes[c]	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-γδ	PG HM-GD-TCR-1; HM-GD-TCR-3	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-8.1, 8.2[c]	PG MM-Vβ-TCR-1	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-6[c]	PG RM-Vβ-TCR-2	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-11	PG RM-Vβ-TCR-3	0.1-100 μg/ml	No	Use plate-bound; direct TCR cross-linking
Staph tox A	TT AT101	1-10 μg/ml	Yes[c]	Vβ-1,3,10,11,17-receptor specificity
Staph tox B	TT BT202; SIG S4881	1-100 μg/ml	Yes[c]	Vβ-3,7,8,17-receptor specificity
Staph tox E	TT ET404	1-10 μg/ml	Yes[c]	Vβ-11,15,17-receptor specificity

[a]Abbreviations: PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; Con A, concanavalin A; Staph tox A, B, & E, *Staphylococcus* enterotoxins A, B, & E.

[b]Supplier addresses and phone numbers are provided in APPENDIX 3. Abbreviations: CAL, Calbiochem; PG, Pharmingen; PH, Pharmacia LKB; SIG, Sigma; TT, Toxin Technology; WD, Wellcome Diagnostics.

[c]When using anti-CD3 and anti-TCR antibodies in soluble form (rather than plate-bound), accessory cells are required. When using Staph enterotoxins, accessory cells must express appropriate MHC class II molecules. Accessory cell dependence is not absolute with anti-Thy-1 antibodies.

Materials

Complete RPMI-5 and RPMI-10 media (*APPENDIX 2*)
Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNIT 3.1*)
Activating agent(s) (Table 3.12.1)
Phosphate-buffered saline (PBS; *APPENDIX 2*)
Accessory cells: unfractionated mouse spleen cell suspension, irradiated or treated with mitomycin C (second support protocol) or T cell–depleted (first support protocol)
[^{3}H]thymidine (*APPENDIX 3*)

15- and 4-ml disposable, polystyrene conical tubes with screw caps
Low-speed centrifuge with Sorvall H-1000B rotor (or equivalent)
1-, 5-, and 10-ml disposable polystyrene pipets
96-well flat- or round-bottom microtiter plates with lids (Costar #3596 or #3799)
25- to 100-µl single- and multichannel pipettors with disposable tips

Additional reagents and equipment for removing organs (*UNIT 1.9*), preparing single-cell suspensions (*UNIT 3.1*), and counting, labeling, and harvesting cells (*APPENDIX 3*)

1. Prepare responder leukocyte suspensions from thymus, spleen, or lymph node in complete RPMI-5 as described in *UNIT 3.1*.

   The size of the intended experiment dictates the number of organs to be collected. See annotation to step 3 for an indication of cell number required, and UNIT 3.1 for number of cells per organ. Spleen, thymus, and lymph node can be used as responder cells, while only spleen is a source of accessory cells. Purified T cells or subpopulations of T cells (i.e., CD4$^+$ or CD8$^+$) cells may also be used. See UNITS 3.1-3.6 for enrichment/depletion methods.

2. Centrifuge single-cell suspensions in 15-ml conical tubes for 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × *g*), room temperature, and discard supernatant.

3. Resuspend cell pellet in complete RPMI-5. Count responder cells and adjust to ~10^6 cells/ml with complete RPMI-10.

   While this concentration (1×10^6 cells/ml or 2×10^5 cells/well) will give satisfactory responses with most cell populations, it is useful to compare 2, 4, and 8×10^5 cells per well in initial pilot experiments. If unfractionated spleen or lymph node cells are used as the responder population, sufficient accessory cells are present and there is no need to supplement the cultures with additional cells. However, if highly purified T cells or T cell subpopulations are used as responders, it will be necessary to add non–T accessory cells depending on the nature of the activating agent (see Table 3.12.1). This is most easily accomplished by adding increasing numbers (0.1, 0.5, and 1.0×10^5) of syngeneic spleen (accessory) cells in 0.1 ml to 2×10^5 T cells in 0.1 ml (see first support protocol). Also, a meaningful comparison of the responsiveness of different cell populations requires titrations of both the activating agents as well as the responding cell populations, and a kinetic experiment.

4. Prepare working solutions of activating agents in 4-ml conical tubes at room temperature as follows. For MAb, toxin, or lectin, make a series of four dilutions from 1 mg/ml stock solutions—e.g., 100, 30, 10, and 3 µg/ml in PBS. For the pharmacological agent, make single dilutions of 100 ng/ml solution of PMA and 1 µg/ml A23187 (or 4 µg/ml ionomycin) in PBS.

   If MAb in supernatant or ascites form are being used, at least four dilutions should also be used. Working solutions should be used immediately, since the various proteins, especially MAb, may bind to the plastic.

See Table 3.12.1 for Vβ specificities of staphylococcal enterotoxins. It is essential to verify that the mouse strain employed expresses the MHC class II surface molecules for which the enterotoxin has a specific binding affinity. See Marrack and Kappler (1989) for further discussion of various enteroxins and their specificities.

5. Add 20 µl of each dilution of activating reagent (MAb, enterotoxin or lectin) to each of three wells of a 96-well flat- or round-bottom microtiter plate. Include control wells with 20 µl of PBS only. Add 20 µl PMA or calcium ionophore at the single concentration indicated in step 4, as the dose-response curve for these agents is extremely narrow.

A series of four dilutions will form one row of each microtiter plate, allowing for efficient organization of the plates.

6. To the wells of the 96-well microtiter plate containing activating agent, add 2×10^5 cells in 0.2 ml.

7. Place microtiter plates in a humidified 37°C, 5% CO_2 incubator for 2 to 4 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions and must be determined empirically (see critical parameters).

8. Add [^{3}H]thymidine to each well. Return the plates to CO_2 incubator to pulse 18 to 24 hr. Harvest cells using a semiautomated sample harvester and measure cpm in β scintillation counter.

9a. Compute the data as the difference in cpm of stimulated (experimental) and control (no activating agent added) cultures. This is done by subtracting the arithmetic mean of cpm from triplicate control cultures from the arithmetic mean of cpm from corresponding stimulated cultures. The results are referred to as "Δ cpm."

9b. Alternatively, compute the data as the ratio of cpm of stimulated and control cultures. This is done by dividing the arithmetic mean of cpm from stimulated cultures by the arithmetic mean of cpm from control cultures. The results are referred to "SI" (stimulation index).

The second method (step 9b) has the disadvantage that small changes in background values will result in large changes in SI and should be interpreted with caution. In most publications, Δ cpm rather than SI values are preferred.

ALTERNATE PROTOCOL

ACTIVATION OF UNPRIMED T CELLS WITH PLATE-BOUND ANTIBODIES

Although it is possible to induce T cell activation with monoclonal antibodies to the CD3/TCR complex in solution during culture, such activation depends on cross-linking of the antibody by Fc receptor–bearing accessory cells. This protocol describes the use of monoclonal antibodies to the CD3/TCR complex by coupling them to the wells of the microtiter plates. The T cell proliferative response induced under these conditions does not require the presence of significant numbers of accessory cells, although the responses obtained may be suboptimal (Jenkins et al., 1990).

Use of this protocol is recommended for use with those antibodies to the CD3/TCR complex which bind poorly to the Fc receptor present on murine accessory cells and which do not induce T cell activation in soluble form. Although all monoclonal antibodies readily couple to plastic under these conditions, it is very difficult to induce a proliferative response with certain antibodies such as the G7, anti-Thy-1 monoclonal antibody. In such cases, the conditions described in the basic protocol should be followed.

Additional Materials

PBS (*APPENDIX 2*), room temperature and 4°C

1 mg/ml purified anti-CD3 or anti-TCR MAb in PBS (for nonspecific activation of T cells) *or* 1 mg/ml purified anti-Vβ or anti-TCR-γδ MAb in PBS (for activation of T cells with specific receptors; see Table 3.12.1)

1. In 4-ml conical polystyrene tubes, prepare a series of four dilutions of MAb from sterile 1 mg/ml stock solutions—e.g., 100, 10, 1, and 0.1 μg/ml—using room temperature PBS.

   Sources and recommended concentrations of monoclonal antibodies can be found in Table 3.12.1; since MAb will bind to plastic, the working dilutions should be used immediately.

   The ability of anti-TCR antibodies to cross-link receptor molecules varies depending on the purity of the MAb preparation and the affinity of the MAb for the TCR/CD3 complex. Optimum dilutions will have to be determined in dose-response experiments. Alternatively, preparations of ascites fluid from the MAb can be tested at different dilutions (e.g., 1:100, 1:200, 1:400, and 1:800), but use of purified antibody will allow for better standardization of the assay.

   Because the efficacy of MAb-induced activation depends on the amount of antibody bound to the bottom of the wells, it is crucial to make the dilutions in a buffer without any additional source of proteins such as FCS or albumin; these would compete with the binding of the antibody, and therefore reduce the responsiveness. For this reason, it is also not recommended to perform the assay with culture supernatants of the appropriate hybridomas.

2. Add 30 μl of each concentration of MAb solution to each of three wells of a 96-well round-bottom microtiter plate. Include control wells of 30 μl PBS only.

   A series of four dilutions will form one row of each plate, allowing for efficient organization of the plates. Consistently better responses are seen with round-bottom (compared with flat-bottom) plates in antibody-mediated experiments.

   Most often, optimal responses are seen with 10 μg/ml antibody. There is no point in adding more than the indicated amount of antibody, since the maximum amount that can bind to surface of the wells is ~2 to 3 μg (A.M.K., unpub. observ.).

3. Cover the plate and gently tap its side to ensure complete covering of the bottom of the wells. Incubate plates 90 min at 37°C. During incubation, proceed to step 4.

   During this incubation, the antibodies bind to the plastic in the wells for subsequent cross-linking of the T cell receptors on responding T cells. Plates can also be prepared the night before an experiment and kept in the refrigerator overnight, after the 37°C incubation.

4. Prepare responder cell suspensions as in steps 1 to 3 of the basic protocol.

   Highly purified T cell populations can be used in these studies as the proliferative response induced is accessory cell–independent. However, the presence of non–T accessory cells does not interfere with the proliferative response.

5. Wash the wells of the incubated plates by adding 200 μl cold PBS and inverting the plates with a flick of the hand on a stack of paper towels placed in a tissue culture hood. Repeat washing procedure two more times to remove excess antibody.

6. To the wells of the washed plates, add ~2×10^5 cells in 0.2 ml.

   If cells are not ready at this stage, plates may be kept in the refrigerator overnight after 100 μl PBS has been added. Presumably, longer storage periods should be acceptable, but our experience is limited to ≤4 day periods. The PBS should be removed before the cells are added.

Most cell populations will give peak responsiveness at this cell dosage, but pilot experiments should be performed to establish optimal conditions.

7. Proceed as in steps 7 to 9 of the basic protocol, but incubate cultures for 2 to 3 days before adding [^{3}H]thymidine.

Kinetic assays should be performed to determine the optimum culture period.

ALTERNATE PROTOCOL

T CELL PROLIFERATION IN MIXED LYMPHOCYTE CULTURES

In the mixed lymphocyte culture (MLC) or reaction (MLR), suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigen (usually MHC class I or class II molecules) expressed on the allogeneic stimulator cells. Responder cells need not be primed because a sufficiently high number of T cells in the MLC will respond to the stimulator population. If the stimulator cell population contains T cells, their uptake of [^{3}H]thymidine must be prevented by irradiation or treatment with mitomycin C; alternatively the stimulator cell suspension can be depleted of T cells (see support protocols).

Additional Materials

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNITS 1.9 & 3.1*) *or* purified T cells or T cell subpopulations (*UNITS 3.1-3.6*)

Stimulator cells: allogeneic mouse spleen cells that differ from the responder cells at *H-2* or *Mls* loci, irradiated or treated with mitomycin C (second support protocol) or T cell–depleted (first support protocol)

1. Prepare responder cell populations as in steps 1 to 3 of the basic protocol. Although unfractionated cell populations can be used as responders in certain situations, it may be preferable to use purified T cells or T cell subsets.

To estimate the MLR of a cell population, it is necessary to perform a dose-response assay with different numbers of responder cells. Typically, three replicate wells are set up containing each of the following: 0.5, 1, 2, and 4 × 10^5 cells (optimal responses are usually obtained with the latter two densities). The setup for these four cell densities will occupy one row (12 wells) of a microtiter plate.

For thymocytes, it may be necessary to use 8 × 10^5 cells per well because the frequency of responding T cells is lower; the lowest number of responder cells could then be 1 × 10^5 and the doses in between would be 2 and 4 × 10^5. Using this range of higher numbers of responder cells may also be preferred when experimental manipulations are expected to reduce the frequency of responding T cells.

2. To a 96-well microtiter plate, add 5 × 10^4 to 4 × 10^5 responder cells in 0.1 ml to each well. For each experimental group, set up three replicate wells.

Stimulation of leukocytes for proliferation in 96-well microtiter plates can be run in parallel with cytotoxic T lymphocyte (CTL) generation (UNIT 3.11), which is performed in 24-well microtiter plates. For example, cells can be diluted to 4 × 10^6 cells/ml and added to 24-well plates in 1.0 ml/well for CTL generation and to 96-well plates in 0.1 ml/well for proliferation.

3. Prepare a single-cell suspension of irradiated or mitomycin C–treated stimulator cells. Alternatively, prepare a suspension of T-cell depleted stimulator cells. Add 0.1 ml to each well of the plates containing responder cells.

The optimum number of stimulator cells must be determined for each MLC and for different responder cells. For a range of responder cells from 0.5-4 × 10^5, test stimulator cells at densities of 2, 4, and 8 × 10^6/ml (i.e., 2, 4, and 8 × 10^5/well). It should be noted that the stimulator cell suspension provides both the specific antigen to be recognized by the responder T cells as well as nonspecific accessory cells. If

highly purified T cells are used as the responder population, it is therefore not necessary to supplement the cultures with non–T accessory cells syngeneic to the responder T cells.

Separate wells with control cultures should be set up that include—for each dose of responder and stimulator cells—replicate wells of responder cells with irradiated or mitomycin C–treated syngeneic stimulator cells. Values obtained from these controls reflect "background" proliferation values (see step 9 of basic protocol). Other negative controls often included are wells with stimulator cells alone and wells with responder cells alone. These are not used for the calculation of the data, but are useful to compare with the background proliferation values; the latter should not be much higher (<2-fold) than those obtained with stimulator or responder cells alone. Higher background values indicate potential autoreactivity.

4. Follow steps 7 to 9 of the basic protocol, but incubate the cultures for 3 to 6 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions, and must be determined empirically (see critical parameters).

DEPLETION OF T CELLS FROM ANTIGEN-PRESENTING/STIMULATOR CELL SUSPENSIONS

SUPPORT PROTOCOL

Although normal unfractionated spleen cell populations can be used as a source of accessory cells, in certain types of experiments it may be preferable to use spleen cell populations from which the T cells have been removed. This procedure ensures that none of the observed proliferative responses of the responder population result from T cell factors derived from the accessory cell population. For example, even T cells whose cell division has been blocked (second support protocol) can produce cytokines. In the following steps, T cell–depleted spleen cell suspensions are prepared using a lytic monoclonal antibody to the T cell antigen, Thy-1. Because almost all the antigen presentation or stimulator cell activity in spleen resides in the non–T cell fraction, this procedure also leads to enrichment of functional antigen-presenting cell function. Further enrichment of antigen-presenting cells (APC) by flotation of the T cell–depleted spleen cells on Percoll gradients is also described. Other procedures leading to enrichment of APC are described elsewhere; the method described in *UNIT 3.7* does not deplete T cells and therefore is not recommended here; the method described in *UNIT 3.15* leads to higher levels of enrichment that are not required in the protocols presented here.

Additional Materials

Spleen cells from nonimmunized mice
Hanks balanced salt solution (HBSS; *APPENDIX 2*)
Low-Tox rabbit complement (Cedarlane #CL3051), reconstituted with ice-cold distilled water and filter-sterilized
Anti-Thy-1.2 ascites (HO-13-4; ATCC #TIB 99) or anti-Thy-1.1 ascites (HO-22-1; ATCC #TIB 100; alternatively, see Table 3.4.1 for other anti-Thy-1 MAb and *UNIT 2.6* for production of ascites)
70% Percoll solution (*UNIT 3.8* and reagents and solutions)

1. Centrifuge the spleen cell suspension derived from single spleen down to a pellet.

The spleen cells should always be from nonprimed animals and should be syngeneic to the responder T cells unless they are to be used as stimulator cells in the MLC.

2. To the pellet, add 0.9 ml HBSS, 0.1 ml complement, and 25 µl anti-Thy-1 ascites.

If cells from more than a single spleen are needed, the procedure should be scaled up accordingly.

The choice of anti-Thy-1 reagent to be used depends on the strain of animal from which the spleen was derived. The great majority of commonly available mouse strains (except AKR) express the Thy-1.2 allele.

3. Incubate the mixture at 45 min in a 37°C water bath.

4. Centrifuge 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × *g*), room temperature, and discard supernatant. Resuspend pellet in HBSS and wash two more times.

5. Count viable cells (*APPENDIX 3*) and resuspend in complete RPMI-10 or PBS for inactivation as in the second support protocol, or in HBSS to prepare low-density accessory cells (see below).

   The T cell–depleted spleen cell population is comprised of B cells, macrophages, and dendritic cells. Further enrichment of cells with enhanced accessory cell function can be obtained by fractionation of this population on Percoll.

6. Dilute 70% Percoll solution to 55% by mixing 23.58 ml of the 70% Percoll with 6.42 ml HBSS. Resuspend T cell–depleted spleen cells from step 5 in HBSS at 20 × 10^6 cells/ml.

7. Layer 3 ml cell suspension over 3 ml of 55% Percoll solution in a 15-ml conical centrifuge tube.

8. Spin 13 min in H-1000B rotor at 3000 rpm (1900 × *g*), room temperature.

9. Remove cells that band at the Percoll/HBSS interface with a 5-in. Pasteur pipet and wash 3 times in HBSS as in step 4.

10. Count viable cells and resuspend in complete RPMI-10 for inactivation according to the second support protocol.

    The population obtained from steps 6 to 10 is comprised of large cells including macrophages, dendritic cells, and activated B lymphocytes. This population of cells is enriched in accessory cell function. When used in either of the basic protocols with purified T responder cells, fewer of the Percoll-purified cells should be needed to provide accessory function.

SUPPORT PROTOCOL

BLOCKING CELLULAR DIVISION OF ACCESSORY/STIMULATOR CELLS

There are two situations in which inhibition of accessory or stimulator cell division should be blocked. When purified T cells rather than unfractionated lymphoid populations are used in the basic protocol, cultures are frequently supplemented with accessory cells syngeneic to the responder T cells. If accessory cell DNA synthesis is inhibited, one can then be certain that the resultant proliferative response is comprised entirely of responder T cells and does not contain a component of recruited B cell proliferation derived from the accessory cell populations. In the MLR, the stimulator cells are spleen cells from mice that differ from the responder cells in *H-2* and/or *Mls* gene expression (see *APPENDIX 1*, Tables A.1C.1 and A.1F.1) and they can also recognize alloantigens on the responder cells. This responsiveness of stimulator cells against responder cells in an MLR (so-called back-stimulation) must be prevented by blocking cellular division. This can be done by treatment of stimulator cells with mitomycin C (a DNA cross-linking reagent) or by g irradiation. Many investigators prefer mitomycin C treatment when antigenic differences encoded for by *Mls* genes are to be measured, or when an irradiation source is not available. For more information on the loci encoding *Mls* genes, see Tables A.1F.2 and A.1F.3.

Mitomycin C Treatment

Additional Materials

Mitomycin C (Sigma #M-0503; store in dark)

1. In a 15-ml aluminum foil–wrapped tube, prepare a solution of mitomycin C in PBS at 0.5 mg/ml and filter sterilize.

   Since mitomycin C is very light-sensitive, it is necessary to prepare a fresh stock solution each day for each experiment.

2. Prepare spleen cell suspension as described in steps 1 and 2 of the basic protocol at a concentration of 5×10^7 cells/ml in PBS.

3. Add mitomycin C to a final concentration of 50 μg/ml (100 μl/ml of cell suspension) and wrap the tube in aluminum foil. Incubate 20 min at 37°C.

4. Add an excess of complete RPMI-5 (i.e., fill tube with ~12 ml) and centrifuge 10 min in Sorvall H-1000B rotor at 1200 rpm ($300 \times g$). Discard supernatant and repeat washing procedure two more times.

   Three washes are crucial, because any traces of mitomycin C left among the cells will reduce proliferative responses when the cells are added to an MLC.

5. Resuspend pellet in complete RPMI-10. Count cells with hemacytometer. Adjust to desired concentration as described in the annotation to step 6 of the basic protocol.

Irradiation Treatment

Prepare a spleen cell suspension as described in steps 1 to 3 of the basic protocol, at a final concentration of $5\text{-}10 \times 10^6$ cells/ml in complete RPMI-10. Using a source of ionizing irradiation (^{60}Co or ^{137}Cs γ-irradiator; e.g., Gammacell 1000, Nordion), deliver 1000 to 2000 rad of irradiation to the cells.

This dose range of irradiation is suitable for most immunologic applications employing spleen cell suspensions. However, antigen presentation by different spleen cells is differentially affected by irradiation (Ashwell et al., 1984): at low doses (500 to 1000 rad), antigen-presenting function of B cells is preserved; after doses of 1100 to 2000 rad, a substantial decline is observed; and doses >2000 rad abolish the participation of B cells as APC. Macrophages and dendritic cells, on the other hand, maintain antigen presentation through doses of 3000 rad. To ensure that B cells do not participate in the responses measured, some investigators prefer to use doses of 2000 rad. However, responsiveness to *Mls* antigens can best be measured with stimulator cells that received doses of <1000 rad, since B cells present *Mls* more effectively. Alternatively, *Mls* responsiveness can be measured after mitomycin C treatment of stimulator cells, since it also preserves the antigen-presentation function of B cells.

When transformed cell lines are used as antigen-presenting or accessory cells, higher doses must be used to ensure blockage of cell division. The appropriate dose will have to be determined empirically for each cell line, but is likely to be at least 5000 rad; some transformed cell lines require as much as 10,000 to 12,000 rad, and may be more sensitive to mitomycin C treatment.

ACTIVATION OF PRIMED T CELLS

BASIC PROTOCOL

Proliferative responses to viruses, protein antigens, minor transplantation antigens, and the male H-Y antigen require in vivo immunization followed by in vitro stimulation. Furthermore, enhanced proliferative responses to those antigens that will generate primary in vitro responses (i.e., MHC antigens) can be obtained by in vivo priming. Multiple immunizations usually elevate in vitro responses.

To immunize animals for in vitro secondary responses to soluble protein antigens or peptides, dissolve antigens and emulsify in complete Freunds adjuvant (*UNIT 2.5*). For strong responses by draining lymph node cells, immunize animals in a hind footpad. For

strong responses by spleen cells, immunize intraperitoneally. Tail-base immunization also can be used as an efficient route of immunization; follow procedure for intradermal injection. To prime animals against cellular antigens, inject intraperitoneally with 1-5 × 10^7 cells that express the antigen. Immunization protocols are described in *UNIT 1.6*.

Within 2 to 3 weeks after in vivo priming, in vitro responsiveness of primed T cells can usually be measured. This assay is often used as a preparation for subsequent in vitro cloning procedures (*UNIT 3.14*) and T cell hybridoma preparation (*UNIT 3.13*).

Materials

Complete RPMI-10 medium (*APPENDIX 2*)
Responder cells: Purified T cells isolated from lymph nodes (*UNITS 3.1-3.6*) of in vivo primed mice
Antigen: 1 mg/ml sterile protein antigen(s) (*UNIT 3.13*), in PBS *or* suspension of irradiated or mitomycin C–treated stimulator cells expressing alloantigens at 8 × 10^6 cells/ml (*UNIT 3.11*, support protocol) in complete RPMI-10 medium (*APPENDIX 2*)
Accessory cells: suspension of irradiated or mitomycin C–treated (or T cell–depleted) spleen cells syngeneic to the responding T cells at 5 × 10^6 cells/ml in complete RPMI-10 medium

4-ml conical tubes
96-well flat-bottom microtiter plates with lids

1. Follow steps 1 to 3 of the first basic protocol for preparation of responder cells.
2. Prepare 4-fold dilution series of the antigens in 4-ml conical tubes, using complete RPMI-10.

   The following dilutions are recommended: 100, 10, 1, and 0.1 μg/ml protein antigens and 8, 4, 2, and 1 × 10^6 cells/ml of stimulator cells in complete medium.
3. Add antigens to 96-well flat-bottom microtiter plates, at 30 μl/well for protein antigens or 100 μl/well for cellular antigens. For each experimental group, set up three replicate wells and include control wells with medium only (no antigen).

   By using four concentrations of antigens and three replicate wells for each dose, one row of a microtiter plate will cover the entire tested range.
4. Add responder T cells in 0.1 ml to each well.

   Purified T cells are recommended; otherwise extremely high background values may be obtained. This appears to be due in part to proliferation of recruited cells (T and non–T) that are not antigen-specific. If unfractionated lymph node cells from recently primed mice are used, add 1-2 × 10^5 cells per well and proceed to step 6.
5. If purified lymph node T cells specific for protein antigens are used, add 0.1 ml of accessory spleen cells syngeneic to the donor of the responder T cells at 5 × 10^5 cells per well.

   Purified T cells require an exogenous source of accessory non–T cells. Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals." They are not required for cell preparations primed against cellular antigens, because accessory cell function is provided by the stimulator cells.
6. Proceed as in steps 7 to 9 of the basic protocol.

   Culture periods before labeling can vary widely and kinetic assays should be performed. In general, for T cells from primed mice, it is likely that the response will peak at day 4 or 5.

REAGENTS AND SOLUTIONS

Percoll solution

Diluent:
45 ml 10× PBS, pH 7.4 (*APPENDIX 2*)
3 ml 0.6 M HCl
132 ml H_2O
Filter sterilize

70% Percoll solution:
63 ml Percoll (Pharmacia LKB #170891-01)
37 ml sterile diluent (above)
Final osmolarity should be 310 to 320 osM

COMMENTARY

Background Information

Proliferative assays for measuring T cell function have certain advantages and disadvantages compared to the cytotoxic T lymphocyte (CTL) assay described in *UNIT 3.11* or the lymphokine production assays in *UNITS 3.15 & 6.3*. Advantages are that proliferative assays are less time-consuming, less labor-intensive, less cell-consuming, and less expensive than "true" effector T cell function assays. A disadvantage is that antigen specificity is not as easily demonstrated in proliferative assays as in CTL assays, unless antigen-specific clones of proliferating cells are used. Furthermore, the proliferative assay only detects dividing cells instead of measuring true effector T cell function.

It is not clear which T cell function is measured in proliferative assays; the proliferative response should therefore be used solely as general indicators of T cell reactivity. Data obtained in proliferative assays might variously reflect proliferation of CTL, lymphokine-producing T cells, or nonactivated "bystander" cells, and will be severely affected by the function of non–T cells such as accessory cells (see below). Since the majority of T cells respond to and produce IL-2 upon activation, differences in responsiveness in a proliferative assay in part reflect differences in IL-2 production by the responding T cells. Proliferative assays therefore become more meaningful when combined with the lymphokine detection assays presented in *UNITS 3.15 & 6.3*. Since responsiveness to IL-2 is also determined by the levels and functionality of IL-2 receptors, further information will be added by including measurements of IL-2 receptors (*UNIT 6.1*) or by flow cytometry (*UNIT 5.4*). Yet, as a first approximation of cellular activation, proliferative assays are valuable.

Critical Parameters and Troubleshooting

Parameters affecting the magnitude of T cell proliferative responses include cell concentration, type of medium, source of serum, incubator conditions (CO_2 level and humidity), type and concentration of activating agent, type of responding T cells, type of accessory/stimulator cells, mouse strain, and culture time. Optimal conditions for individual laboratories and experiments must be derived empirically with respect to these variables, but general guidelines are provided below.

A number of agents can be employed in the first basic protocol to induce T cell proliferation (Table 3.12.1). T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex—i.e., anti-CD3, anti-TCR-αβ or -γδ, as well as anti-Vβ antibodies that are capable of interacting with a subset of cells bearing a specific TCR. A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" (Marrack and Kappler, 1989) because they stimulate T cells via the variable (*V*) gene segment of the TCR. Different toxins have affinities for different Vβ chains and these specificities make them valuable reagents for activating T cells. The activating capacity of toxins is also dependent on their ability to bind to MHC class II molecules (i.e., responding T cells react with the toxin/class II complex); thus, responsiveness varies with the

mouse strain used. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of action of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators (Gunter et al., 1984).

When comparing the reactivity of different cell populations, it is essential to perform dose-response assays for responder T cells and activating agents and for both responder and stimulator T cells (in MLR), since each population may yield optimal responses at different cell numbers. This may reflect differences in frequency of responding cells, and hence may indicate a need to perform limiting dilution assays (*UNIT 3.15*). Since peak responsiveness of different populations of T cells may occur at different times, it is also essential to perform kinetic experiments—i.e., compare responsiveness at days 2, 3, 4, and 5.

Differences in responsiveness need not necessarily be due to differences in the frequency of responding T cells, but may also indicate differences in the efficacy with which co-stimulatory activity or "second signals" are delivered by the accessory cells present in different cell populations. The type of interactions pertinent to the generation of primary responses by T cells is explained in the commentaries of *UNITS 3.8, 3.11, & 3.13*. Specific requirements for inducing activation with immobilized antibodies have been described (Staerz and Bevan, 1986; Hathcock et al., 1989; Jenkins et al., 1990). A responding cell population completely devoid of accessory cells (such as purified populations of splenic or lymph node T cells or cloned T cells) will yield fine responsiveness in an MLC, since accessory cell function is provided by the stimulator cells; however, the same population will generally not yield responses when mitogens, antigens, or enterotoxins are used. In such a setting, accessory cells may also function as antigen-presenting cells (APC). Addition of irradiated or mitomycin C–treated syngeneic sources of accessory cells (either whole spleen cells or purified APC; see first support protocol) can be used to restore responsiveness in purified T cells. The need for accessory cells can sometimes be bypassed when anti-TCR monoclonal antibodies are coupled to plastic, or when certain anti-Thy-1 monoclonal antibodies are used; however, these conditions do not necessarily result in optimal responsiveness (Jenkins et al., 1990).

The level of [^{3}H]thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA and "cold" thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of nonactivated cells will also interfere with the accuracy of this last parameter.

The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in [^{3}H]thymidine incorporation values. When values obtained in triplicate cultures correspond poorly (e.g., >5% difference in cpm values >1000), technical problems such as cell clumping, dilution, and pipetting should be considered. Excessively high values may be obtained from contaminated wells, as [^{3}H]thymidine will be incorporated into replicating bacteria; therefore, it is good practice to check the wells from microtiter plates under an inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes.

It is also useful to check for blast formation by microscopic examination of the cultures: activated lymphocytes will tend to enlarge, and detection of blasts will give a general indication of successful activation.

The main problem that may occur with proliferative response assays is high levels of background [^{3}H]thymidine incorporation in control cultures without antigens. This problem is frequently due to the fetal calf serum (FCS) used to supplement the cultures, which may be mitogenic for B cells. Different lots of FCS should be screened to select those that are nonstimulatory or only weakly stimulatory in the absence of other stimuli, and that support strong proliferative responses upon antigenic stimulation of T cells.

If flat-bottom microtiter plates are used in the procedure and weak responses occur, it may be useful to switch to round-bottom plates. Our laboratory has found consistently better responses in round-bottom plates when

thymocytes are used as responder cells or with slight alloantigenic differences between responding and stimulating cells. In addition, antibody-mediated experiments yield better results with round-bottom plates. Presumably, this reflects better cell contact obtained in such plates; optimal responses will almost certainly occur at different cell numbers than in flat-bottom plates and densities will have to be adjusted accordingly.

Although satisfactory responses to most alloantigens can be obtained with complete RPMI-10 medium, it may be necessary to compare different media. This need arises when the proliferative responses are weak (i.e., when [^{3}H]thymidine values for activated cultures are <10-fold higher than those for control cultures) and may occur under various circumstances: weak alloantigenic differences between responder and stimulator cells, weak T cell proliferative function in the responder cells or diminished APC function in the stimulator cells due to experimental manipulations, or a low precursor frequency of responding T cells. Thymocytes in particular do not contain a high level of responding T cells. Frequently, proliferation can be improved when complete Clicks or Dulbeccos media are used (with additives as described in *APPENDIX 2*), presumably because these media contain additional nutrients and have an osmolarity more compatible with mouse serum than RPMI.

When RPMI is used as medium, 5% CO_2 will be sufficient, but for other media, a 7.5% CO_2 concentration in the incubator will be more satisfactory. Generally, the buffering capacity of DMEM is insufficient at 5%, but fine at 7.5%. Much will also depend on the proliferative activity of the responding population of T cells (e.g., vigorous proliferation will reduce the pH in the cultures); it is therefore recommended to compare responsiveness in initial pilot experiments in incubators set at different CO_2 concentrations.

The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses, such as those obtained with thymocytes or a weak alloantigenic difference, will require a longer culture period (5 to 6 days) than those which elicit a higher frequency of responding T cells (3 to 4 days). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell proliferation. Addition of [^{3}H] thymidine on days 2, 3, 4, 5, and 6 will provide a useful test; further extension of the culture period will not yield any improvements, due to exhaustion of nutrients in the medium.

Anticipated Results

For proliferative assays described in the basic protocol, which activate the majority of the responding T cells, responses of 100,000 cpm should be obtained; in the MLR or following activation with monoclonal antibodies to subpopulations of T cells (anti-Vβ), responses up to 100,000 cpm may be observed; however, measurements of 20,000 cpm (with tight standard errors) can be quite satisfactory. Background values of <1000 cpm should be expected. Reported results (as described in step 9a) should be mean cpm of experimental wells minus background cpm (Δ cpm).

Time Considerations

The time required to set up proliferative assays is not more than a day, with the number of hours depending on the number of different groups of responder cells that must be prepared. The time required for incubation of cells ranges from 2 to 6 days, as noted above in critical parameters. Following an additional 18- to 24-hr incubation period for pulsing, harvesting the cells and measuring cpm will require several hours depending on the number of plates (~15 min for harvesting each plate and ~100 min for counting each plate at 1 min/sample).

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Details the MLC proliferation assay.

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EXHIBIT B

Dendritic cells are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

The Dendritic Cell Advantage: New Focus For Immune-Based Therapies

by Ralph M. Steinman

Summary

Dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. The best-studied function of DCs is to convert antigens into immunogens for T cells. The "DC advantage" entails a mynad of functions. DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity. Another special feature of DCs is their location and movement in the body; DCs are stationed at surfaces where antigens gain access to the body. The events that make up the life history of DCs are now being unraveled in molecular terms. As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent.

The focus of immune therapeutics has been on lymphocytes, the cellular mediators of immunity, and the suppression of lymphocyte function. The drug ciclosporin (cyclosporine) is an excellent and successful example. However, medicine needs therapies that enhance immunity or resistance to infections and tumors. Medicine also needs strategies, whether suppressive or enhancing, that are specific to the disease-causing stimulus or antigen. In contrast to lymphocytes, dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. DCs capture antigens and then initiate and control the activities of lymphocytes, including the development of resistance to infections and tumors (reviewed in references 1–3).

The controlling role of DCs is best known for thymus-dependent lymphocytes or T cells which are important in many diseases, the most poignant being the AIDS epidemic (Table I). DCs were identified in a few laboratories that were focusing on the induction of immunity from resting T cells. It was noted that immune tissues (spleen, lymph nodes, lymph, blood) had a small fraction of cells with unusual "tree-like" or "dendritic" processes. These distinctive cells had not been recognized previously and they proved to have distinct functions. Most importantly, DCs were potent inducers of immunity even in animals, not just the test tube, and now even in patients (reviewed in references 1–3).

The DC field was held back by the fact that there were so few cells relative

TABLE I: HUMAN DISEASES THAT INVOLVE T CELLS

- Rejection of organ transplants and graft-vs.-host disease in bone marrow transplantation
- Resistance to many infections including vaccine design
- Vaccines against tumors and immune therapies for existing tumors
- Allergy
- AIDS
- Autoimmune diseases like insulin-dependent (juvenile) diabetes, multiple sclerosis, rheumatoid arthritis and psoriasis

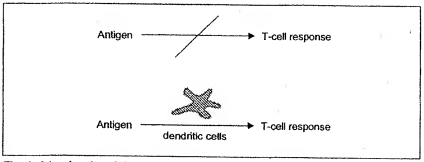

Fig. 1. A key function of dendritic cells. Antigens within tumors, transplants and infectious agents need to be presented by DCs to become immunogens, i.e., to make T cells begin to grow and exhibit their helper and killer functions.

to other players in the immune system such as B cells, T cells and macrophages. In reality, DCs are quite abundant for the job they have to do, namely, to initiate immune responses from antigen-specific T cells. In immune system organs like lymph nodes, DCs form an extensive network throughout the T cell-rich regions and physically outnumber any given antigen-reactive T cell by at least 100 to 1. The DC field was also held back because many thought that the cells were no different from macrophages, thus keeping investigators from working on the active DCs. In reality, DCs were identified on the basis of profound differences from macrophages, and their many distinct properties and functions were only uncovered by separating DCs from macrophages.

The best-studied function of DCs is to convert antigens into immunogens for T cells. The antigen receptors on T cells do not focus on intact proteins in microbes and tumors, but instead recognize fragmented or processed proteins, that is, peptides. The processing of protein antigens into peptides occurs within cells, and then the peptides are displayed or presented at the cell surface affixed to products of the major histocompatibility complex (MHC). The ensuing interaction between a T-cell receptor (TCR) and its specific MHC–peptide complex allows a T cell to detect peptides formed within cells in transplants, tumors, sites of infection and self tissues attacked during autoimmune disease (Table I). "Antigens" refers to specific substances recognized by the immune system, while "immunogens" refers to antigens that effectively induce responses either by themselves or together with enhancing materials called "adjuvants." For T cells in particular, antigens and immunogens are not one and the same (Fig. 1). Even preprocessed peptides and MHC–peptide complexes are weak immunogens. This was evident early on in the work of Peter Medawar, the great scientist who discovered the immune basis of transplantation. He spent many years trying to purify functioning transplantation antigens. These efforts were to little avail.

What was not known in Medawar's time is that transplantation antigens (later shown to be MHC–peptide complexes) become immunogenic when presented by DCs.[4] In other words, transplantation antigens when presented on many cell types are weak immunogens, but on DCs they become powerful inducers of immunity.[4] The same is true of peptides that become much more immunogenic when presented on DCs. DCs activate T cells by getting them to divide and express their helper and killer functions. Then the activated T cells interact with other antigen-presenting cells to eliminate the antigen in question. DCs are also called "nature's adjuvant," because prior adjuvants were artificial substances used to enhance immunity. The DC advantage entails a myriad of functions, some of which will be considered below.

Potency of dendritic cells in initiating immunity in tissue culture

What are some specific features of DCs that warrant attention? The first is their potency. Very small numbers of DCs are sufficient to trigger strong T-cell responses in test tubes. Immune assays are generally carried out with impure antigen-presenting cells, applied at a dose of one presenting cell for every T cell, the latter often preactivated. In contrast, roughly one DC per 30–100 T cells is more than sufficient to induce optimal responses, including responses by resting T cells. A single DC can simultaneously activate 10–20 T cells nestled within its sheet-like processes. Therefore, DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity.

It has always been clear that the accessory function of DCs did not depend exclusively on their capacity to process antigens to form MHC–peptide complexes. This is because the stimuli that were used to define the potency and immune-activating role of DCs did not require that the DCs process an applied antigen. Such stimuli included major transplantation antigens, mitogens, contact allergens, anti-

T-cell antibodies and superantigens. Furthermore, once resting T cells were activated by DCs, the T cells responded vigorously to antigens presented by other cell types, showing that the latter were not deficient in forming ligands for the antigen receptor on T cells, but instead lacked accessory properties.

The word "accessory" has since been replaced by the terms "professional" and "co-stimulatory," but the basic concept is unchanged by shifting terminology. T cells need stimuli other than their specific trigger or ligand (MHC–peptide complexes) to begin to grow and function, for example, to produce the interleukins and killer molecules mentioned above. DCs are potent in providing the needed accessory or co-stimulatory functions. For example, DCs produce an adhesion molecule called DC-SIGN that binds to a target on resting T cells called ICAM-3,[5] and DCs express very high levels of a stimulatory molecule called CD86 that binds to CD28 on resting T cells.[6] These are but two examples of the specialized activities of DCs. These cells do not operate as a single magic bullet.

Position of dendritic cells *in vivo*

Another special feature of DCs is their location and movement in the body. As criteria were developed to identify DCs, it became feasible to go back into the animal and patient to look for the corresponding cells in different tissues. DCs are stationed at surfaces where antigens gain access to the body (Fig. 2, left). The skin and the airway have been the best studied. DCs are found in afferent lymphatic vessels, special channels that allow cells to move from peripheral tissues to lymphoid organs, primarily the T-cell areas (Fig. 2, middle and right). This migration is most readily observed in models of skin transplantation and contact allergy, which are the two most powerful immune responses known.

DC migration is likely to be very important. The body's pool of T cells primarily traffics through the T-cell areas of lymph nodes, rather than through tissues where antigens are usually deposited. So when DCs capture antigens in the skin, airway or another peripheral tissue, their migration to the T-cell areas gives them a chance to select the corresponding rare specific T cells from the assembled repertoire (Fig. 2). The selected T cells then increase in numbers (clonal expansion) and function, enabling the specific immune response to begin. The initial frequency of T cells that recognize an antigen is very small. Only one in 10,000–100,000 of T cells in the repertoire responds to a specific MHC–peptide complex. Therefore, it is so precise and efficient for DCs to be able to pick up an antigen in the periphery and then initiate the immune response from rare T-cell clones in lymphoid organs.

The events that make up the life history of DCs (Figs. 2 and 3) are now being unraveled in molecular terms. For example, scientists are figuring out how to expand antigen-capturing precursors to DCs using flt3 ligand and granulocyte colony-stimulating factor (G-CSF). Key players for the mobilization of DCs from the periphery to lymph nodes are the multidrug resistance receptors, usually studied for their capacity to mediate resistance to chemotherapeutic agents rather than movement of DCs. Migration of DCs is controlled by chemokines produced in the lymphatic vessels and lymphoid organs (Fig. 2). These act on DC chemokine receptors to orchestrate their movement to the T-cell areas. Then within the lymphoid tissue, several members of the tumor necrosis factor (TNF) and TNF-receptor families, such as TRANCE and CD40 ligand, trigger DC production of cytokines like interleukin-12. The TNF family also maintains DC viability. Otherwise the cells die within a day or two. Each of these components of DC function provides targets for manipulating immunity.

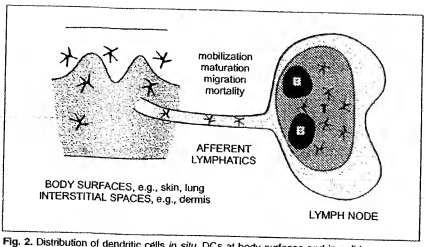

Fig. 2. Distribution of dendritic cells *in situ*. DCs at body surfaces and in solid organs can pick up antigens, move to the lymphoid tissues to find antigen-specific T cells and initiate immunity. Molecular mechanisms are being uncovered that govern the mobilization, maturation, migration and mortality of these DCs. In the lymph node, T lymphocytes are selected for expansion and differentiation into helper and killer T cells. The activated T cells then leave the lymph node to return to the body surface or peripheral organ to eliminate the antigen.

Priming of T-cell immunity via dendritic cells

Animal studies

During the early research on DCs, several labs administered antigens to experimental animals and then tried to identify the cells that had captured the antigens in a form that was immunogenic. Regardless of the route of antigen administration (blood, muscle, skin, intestine and airway), DCs were the major reservoir of immunogen.

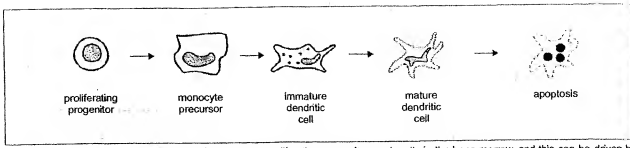

Fig. 3. The life history of dendritic cells. DCs arise from proliferating progenitors, primarily in the bone marrow, and this can be driven by cytokines like flt-3 ligand and G-CSF. Precursors are formed, such as the monocytes in blood, which then give rise to immature DCs. The immature DCs are capable of producing large amounts of antigen-presenting MHC products and capturing antigens. Multidrug resistance receptors are newly recognized players in the mobilization of immature DCs. DCs mature in response to various stimuli such as infection and inflammation, and migrate under the influence of chemokines to the lymphoid tissues. There the DCs die within a day unless their life-span is prolonged by TNF-family molecules expressed by the activated T cells.

Next, DCs were used as nature's adjuvant to immunize animals. The DCs were taken from mice or rats, exposed to antigens *ex vivo* and injected back into immunologically naive recipients. The animals became immunized to the antigens that had been captured by the DCs, and the immunization took place in the lymph nodes draining the site of DC injection. Genetic proof was provided that the DCs were priming the animal directly and not simply handing off their antigen to other cells.[7,8]

DC-based immunization is really very different from all prior attempts at cell therapy. Immunology has had extensive experience with "passive immunization," whereby a recipient is given large numbers of cells that are activated prior to injection. It is hard to produce such large numbers of cells, and their lifespan, diversity and efficacy are all finite. In contrast, when relatively small numbers of antigen-charged DCs are used to induce immunity, this produces "active immunization." Now the animals (and patients, see below) can make their own diverse and longer lasting immune response to the antigen-bearing DCs.

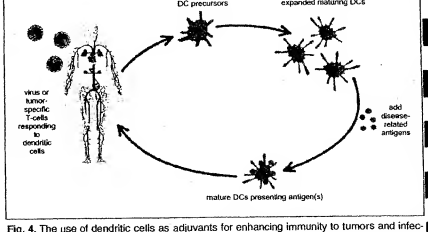

Fig. 4. The use of dendritic cells as adjuvants for enhancing immunity to tumors and infectious agents in humans. This new form of immune therapy begins with the isolation of DC precursors from the patient, usually from blood. The precursors develop *ex vivo* (in relatively simple tissue culture systems) into large numbers of more mature DCs. During this time, the DCs are charged with antigens from the tumor or infection. Then the DCs are reinfused to elicit immunity and thereby resistance to the disease.

Human studies

The above experiments made it clear that DCs, pulsed *ex vivo* with antigens, actively immunized animals and raised the exciting possibility that scientists would be able to induce resistance to tumors, infections and transplants in patients. For example, could one expose patients' DCs *ex vivo* to antigens in their tumors and then reinfuse the antigen-bearing DCs to elicit tumor-specific immunity (Fig. 4)? This approach is actually not terribly complicated, but one first had to overcome a major obstacle and learn to generate large numbers of DCs. These techniques became available in the 1990s. They have energized the field and, accordingly, clinical trials for the immunization of humans against cancer have begun on most continents.

It is evident that DCs can serve as adjuvants for humans, converting antigens into immunogens.[9,10] Even in advanced cancer, immune responses already have been observed that are similar to or better than immune responses obtained with other approaches. However, this approach is still in its preliminary stages, since a good deal of science remains to be developed. On the one hand, there are critical unknowns in terms of overall DC biology. Many of the clinical studies to date, for example, have overlooked key features that could improve DC function, such as the need for DCs

to be sufficiently mature (see below) to be effective *in vivo*. Also, DC biology has to be placed in the context of specific tumors and pathogens and patients for DC-based therapies to be optimized.

To summarize and further illustrate the role of DCs in the context of human disease (Table I), consider the need to harness T cells to resist tumors and chronic infections. Protein antigens often are known for a tumor-like melanoma, or for a virus like HIV-1 whose genetic sequence has been available for more than 15 years. However, this knowledge about antigens from melanoma and HIV-1 antigens remains to be converted into methods that provide better immunogens either for immune therapy of melanoma or for the design of HIV-1 vaccines. This is because some important facts of immunological life are being overlooked. When antigens are injected, they also need to gain access to the right DCs to become immunogens (Fig. 1).

Delivering antigens to dendritic cells

Broadly speaking, a central goal is to learn how to deliver or "target" antigens to DCs and simultaneously to differentiate or "mature" the cells to their most potent state. These two challenges, antigen targeting and DC maturation, prove to be intertwined.

Targeting means that the antigen should be in a form that the DCs can recognize. Without such recognition, the uptake and subsequent processing of antigen to form MHC–peptide complexes is suboptimal. DCs have a number of special mechanisms for capturing antigens and converting these into MHC–peptide complexes (Table II). For example, DCs have a receptor called DEC-205 whose binding partners or ligands are still unknown. Nonetheless, it is clear that DEC-205 greatly increases the capacity of DCs to form MHC–peptide complexes.[11] DCs also carry out a fascinating process called "cross-presentation." DCs can take up dying cells and efficiently extract peptides from them, so antigens "cross" from the dying cell to the DC. The discoverers of this phenomenon called it "resurrecting the dead."[12] Cross-presentation allows DCs to efficiently form MHC–peptide complexes from dead cells in tumors, transplants and tissues under autoimmune attack.

TABLE II: DENDRITIC CELL SPECIALIZATION TO INCREASE MHC–PEPTIDE COMPLEX FORMATION

- Receptors for antigen uptake, e.g., DEC-205
- Processing of dying cells, nonreplicating microbes and immune complexes onto MHC class I ("cross-presentation")
- Regulation of antigen processing by maturation stimuli
- Clustering of T-cell receptor ligands with co-stimulators like CD86

Special uptake and processing mechanisms allow DCs to tailor a protein antigen, as well as the proteins in a complex microbe or tumor cell, into peptides that bind to an individual's MHC products. The latter are exceptionally polymorphic, differing genetically from one individual to another. As a result, the relevant immunizing peptides differ from one individual to another. One reason why peptides are not ideal immunogens is that they must be individualized. DCs, in contrast, can capture antigens with high efficiency and likewise extract peptides that are relevant for any individual.

A second DC advantage is that these cells have the many required accessory or co-stimulatory properties for converting the selected peptides ("antigens") into effective immunogens. A third DC advantage is that these cells position themselves in a way that leads to the identification of rare antigen-reactive T lymphocytes *in vivo* (Fig. 2). DCs thus overcome many of the difficult obstacles in initiating immunity.

In order for an antigen to be a strong immunogen, one needs to provide a stimulus for the final differentiation or maturation of the DCs (Fig. 3). Most DCs in the body are in an immature state and lack many features that lead to a strong T-cell response. Immature DCs, for example, lack the CD86 and CD40 molecules that greatly boost the DC–T cell interaction. Immature DCs also lack a chemokine receptor called CCR7 that seems very important for proper migration and homing to lymph nodes to start immunity. For cancer immunology, it is unlikely that tumors provide maturation stimuli. Tumors may even block DC maturation induced by other stimuli. Therefore it is important to learn how to deliver tumor cells to DCs and bypass the normal obstacles to effective antitumor immunity.

Surprising recent evidence actually links DC maturation to the efficient formation of MHC–peptide complexes or TCR ligands (Table II). Immature DCs take up antigens, but they do not make abundant MHC–peptide complexes until they receive a maturation stimulus.[13,14] Maturation also up-regulates CD86 co-stimulators, but the CD86 actually travels together with the TCR ligands to the surface of the DCs. At the DC surface, the MHC molecules and CD86 remain clustered with each other, keeping the machinery for T-cell activation juxtaposed. This phenomenon will help explain the potency of DCs, because TCR ligands and co-stimulators are displayed together on the cell surface and in high levels.

Control points beyond antigen targeting and maturation of DCs

Research on DCs is moving more vigorously, because the cells are more readily available and because their role in the immune system is considered essential. Nonetheless, researchers in this field are just beginning to find ways to manipulate DCs *in situ*. Putting together an antigen that targets

to DCs with a stimulus for DC maturation will be a major step in improving the conversion of antigens into immunogens, as in immune-based therapies against tumors and infectious agents.

Additional challenges and questions are evident:

- How can DC numbers be increased *in situ* and how can active DCs be mobilized to a cancer or site of chronic infection?
- Can DCs induce strong immune memory to make vaccination long lasting and effective (we have only been reviewing the role of DCs in the initiation of immunity)?
- Can DCs change the quality of the immune response? "Quality" refers to recent evidence for different types of DCs, especially a subset that induces Th1-type T cells for resistance to infectious agents and strong memory.
- Is it possible to move beyond DC-based immunization experiments and use DCs to either regulate or tolerize the immune system, as frequently required in transplantation and autoimmune diseases?
- Can DCs influence elements of the immune system other than T cells; for example, B cells and the innate defenses provided by natural killer (NK) and NK-T cells?

The answer to all these questions is a preliminary "yes." As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent.

Dendritic cells and better control of disease

DCs provide important avenues for the investigation of human disease. Many labs are exploiting DCs to identify antigens relevant for immunity against human pathogens. In these experiments, one introduces complex but clinically important antigens to DCs and then identifies which components are best presented to the immune system. We have recently used this approach to identify previously unknown immune responses to the Epstein-Barr virus,[15] a virus we all carry that has the potential to cause cancer like Hodgkin's lymphoma. Other laboratories have been using DCs to identify new antigens in other infectious agents, in transplants and in cancers like melanoma.

Investigators are also manipulating DCs *ex vivo* and then reinfusing the cells to identify conditions leading to strong immunity in patients (Fig. 4). In particular, DC-mediated active immunization against cancer is being vigorously pursued, as mentioned above. Instead of manipulating DCs *ex vivo*, a more desirable goal would be able to alter DCs directly *in situ*. Some approaches are under way. An example is the injection of cytokines like flt3 ligand and G-CSF to mobilize various precursor populations of DCs. One should also develop methods to control DC mobilization, migration and maturation. In sum, DCs are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

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Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4143–4147, May 1991
Immunology

EXHIBIT C

Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor

(heterodimeric lymphokine/T-cell growth factor/lymphokine-activated killer cells/coordinate gene regulation/interleukin-12)

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Communicated by John J. Burns, February 4, 1991

ABSTRACT **Cytotoxic lymphocyte maturation factor (CLMF) is a disulfide-bonded heterodimeric lymphokine that (*i*) acts as a growth factor for activated T cells independent of interleukin 2 and (*ii*) synergizes with suboptimal concentrations of interleukin 2 to induce lymphokine-activated killer cells. We now report the cloning and expression of both human CLMF subunit cDNAs from a lymphoblastoid B-cell line, NC-37. The two subunits represent two distinct and unrelated gene products whose mRNAs are coordinately induced upon activation of NC-37 cells. Coexpression of the two subunit cDNAs in COS cells is necessary for the secretion of biologically active CLMF; COS cells transfected with either subunit cDNA alone do not secrete bioactive CLMF. Recombinant CLMF expressed in mammalian cells displays biologic activities essentially identical to natural CLMF, and its activities can be neutralized by monoclonal antibodies prepared against natural CLMF. Since this heterodimeric protein displays the properties of an interleukin, we propose that CLMF be given the designation interleukin 12.**

The molecular cloning and expression of recombinant cytokines has made possible both significant advances in our understanding of the molecular basis of immune responses and the development of new approaches to the treatment of disease states. As an example, recombinant interleukin 2 (recombinant IL-2) has been shown to be capable of causing regression of established tumors in both experimental animals (1) and in man (2); however, its clinical use has been associated with significant toxicity (2). One potential approach to improving the therapeutic utility of recombinant cytokines is to use them in combination (3, 4). With this concept in mind, we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 *in vivo*. This led to the identification of a factor, designated cytotoxic lymphocyte maturation factor (CLMF), that synergized with recombinant IL-2 to facilitate the generation of both cytolytic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells *in vitro* (5, 6). CLMF was subsequently purified to homogeneity from a human lymphoblastoid B-cell line (NC-37) and was shown to be a 75-kDa disulfide-bonded heterodimer composed of two subunits with molecular masses of 40 kDa and 35 kDa (7).¶ We now report the molecular cloning and expression of CLMF.

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MATERIALS AND METHODS

cDNA Cloning. A subline of NC-37 cells selected for its ability to produce high levels of CLMF (7), NC-37.98, was induced with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 for 16 hr. Poly(A)$^+$ RNA was isolated, and random hexamer-primed cDNA libraries were established in phage λgt10 by standard procedures. Mixed-primer polymerase chain reaction (PCR) using controlled ramp times (8) was performed as follows. PCR primers contained all possible codons and were 14 or 15 nucleotides long (Fig. 1) with a 5′ extension of 9 nucleotides containing an *EcoRI* site for subcloning. Degeneracies varied from 1 in 32 to 1 in 4096; 0.5–4 pmol per permutation of forward and reverse primer was used in a 50- to 100-μl PCR mixture with 40 ng of cDNA made from NC-37.98 cells that had been activated by culture with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml for 16 hr (40-kDa subunit) or with 3 μg of human genomic DNA (35-kDa subunit). PCR cycling parameters were as follows. Initial denaturation was at 95°C for 7 min. Low-stringency annealing was performed by cooling to 37°C over 2 min, incubating 2 min at 37°C, heating to 72°C over 2.5 min, extending at 72°C for 1.5 min, heating to 95°C over 1 min, and denaturing at 95°C for 1 min. This cycle was repeated once. Thirty standard cycles (40-kDa subunit) or 40 standard cycles (35-kDa subunit) were performed as follows: 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. Final extension was at 72°C for 10 min. "Amplicons" of the expected size were gel-purified, subcloned, and sequenced. The 40-kDa subunit cDNAs were isolated by hybridizing the 54-mer amplicon in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 20% formamide at 37°C overnight. Filters were washed in 2× SSC at 42°C for 30 min and exposed to x-ray film. The 35-kDa subunit cDNAs were isolated by hybridizing the 51-mer amplicon in 5× SSC/20% formamide at 37°C overnight. The filters were washed in 2× SSC at 40°C for 30 min and exposed to x-ray film. Positive clones were plaque-purified, their inserts were subcloned into the pBluescript plasmid, and their sequences were determined by using Sequenase.

Expression. cDNAs were separately engineered for expression in vectors containing the simian virus 40 early promotor essentially as described (9). COS cells were transfected with both CLMF subunit expression plasmids mixed together or

Abbreviations: CLMF, cytotoxic lymphocyte maturation factor; rCLMF and nCLMF, recombinant and natural CLMFs; CTL, cytolytic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; n, natural; PCR, polymerase chain reaction.

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¶The cDNA sequences reported in this paper have been deposited in the Genbank data base [accession nos. M38443 (35-kDa CLMF subunit) and M38444 (40-kDa CLMF subunit)].

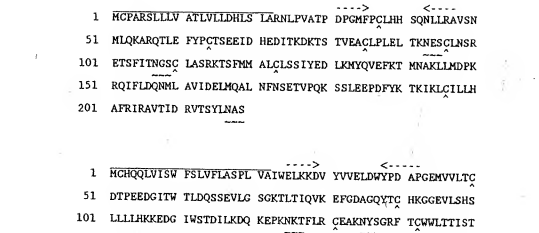

FIG. 1. Amino acid sequences of the 35-kDa (*Upper*) and 40-kDa (*Lower*) CLMF subunits as deduced from the respective cDNAs and shown in single-letter code. Signal peptides are overlined, cysteine residues are marked by a caret, and N-linked glycosylation sites (NXS, NXT, where X is another amino acid) are underlined. The peptide sequences used to generate PCR probes are overlined with arrows indicating the direction of amplification.

with each one separately by the DEAE-dextran method. Twenty-four hours after transfection, the serum-containing medium was replaced with medium containing 1% Nutridoma-SP (Boehringer Mannheim), and supernatant fluids were collected from the cultures after 40 hr. These fluids were stored at 4°C until tested in the bioassays.

General Methods. Standard molecular biological procedures were used as described (10). CLMF bioassays were performed as detailed (7).

Computer Searches. The National Biomedical Research Foundation protein data base (Release 26.0) as well as the Genbank and European Molecular Biology Laboratory databases (Releases 65.0 and 24.0, respectively) were searched for sequences homologous to CLMF cDNAs. The two subunit sequences were compared to each other using the ALIGN program (mutation data matrix, break penalty of 6; see ref. 11).

RESULTS

Partial N-terminal amino acid sequences of the two CLMF subunits (7) were used to generate completely defined 51- to 54-base-pair (bp)-long oligonucleotide probes by means of mixed primer PCR. These probes were used to screen cDNA libraries made from RNA from activated NC-37.98 cells, and cDNAs encoding the two subunits were isolated and characterized. Both cDNAs encode secreted proteins with a classical hydrophobic N-terminal signal peptide immediately followed by the N terminus of the mature protein as determined by protein sequencing (7). Two independent cDNA clones for the 40-kDa subunit were shown to be identical. Both encode the mature 40-kDa subunit that is composed of 306 amino acids (calculated M_r = 34,699) and contains 10 cysteine residues and four potential N-linked glycosylation sites (Fig. 1). Two of these sites are within isolated tryptic peptides derived from the purified 40-kDa CLMF subunit protein. Amino acid sequence analysis has shown that Asn-222 is glycosylated, whereas Asn-125 is not (Fig. 1; F. Podlaski, personal communication). The mature 35-kDa subunit is composed of 197 amino acids (calculated M_r = 22,513), with 7 cysteine residues and three potential N-linked glycosylation sites (Fig. 1). When the purified CLMF is reduced with 2-mercaptoethanol and analyzed by SDS/PAGE, the 35-kDa subunit appears to be heterogeneous, suggesting that it may be heavily glycosylated (7). Two variants of 35-kDa subunit-encoding cDNAs were isolated. The first type had the sequence shown in Fig. 1. Additional isolates contained what is probably an allelic variation, replacing Thr-213 with a methionine residue.

Computer searches of sequence databases showed that the amino acid sequences of the two subunits are not related to any known protein. The subunit sequences are also not related to each other, since a comparison using the ALIGN program (11) gave a score of 1.27; only scores >3 are considered to indicate significant evolutionary relationship (12). The genes encoding the subunits appear to be unique, since low- and high-stringency hybridizations of genomic blots revealed identical banding patterns (data not shown). RNA blots showed the size of the 40-kDa subunit mRNA to be 2.4 kb, whereas the 35-kDa subunit was encoded by a 1.4-kb transcript (Fig. 2). Expression of the two mRNAs encoding the subunits was coordinately regulated upon induction (Fig. 2). When NC-37.98 cells were activated with PMA and calcium ionophore for 72 hr, mRNA encoding each of the CLMF subunits was minimally detectable at 6 hr after the beginning of induction but was readily detected at 24 hr and continued to accumulate until maximal levels were reached at 72 hr (normalized to GAPDH mRNA levels; see the legend to Fig. 2). In contrast, the mRNA for IL-2 in activated NC-37.98 cells was already at high levels at 6 hr and subsequently decreased, whereas the mRNAs for the low-affinity IL-2 receptor (p55) followed the induction pattern seen for the CLMF subunits. Scanning of RNA blots also revealed that steady-state mRNA levels for the 40-kDa

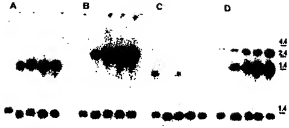

FIG. 2. RNA blots showing the coordinate induction of the 35-kDa (*A*) and 40-kDa (*B*) CLMF subunit mRNAs and IL-2 mRNA (*C*) and its p55 receptor mRNA (*D*). Poly(A)$^+$ RNA (5 μg) from NC-37.98 cells activated with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml were loaded in each lane. Lanes from left to right in each panel show RNAs isolated 6, 24, 30, 48, and 72 hr after induction, respectively. (*Upper*) Four-day exposures. (*Lower*) Two-hour exposure of the same blots after stripping and rehybridization with a GAPDH probe. Marker sizes are in kb (BRL RNA ladder).

CLMF subunit were severalfold higher than those for the 35-kDa subunit expressed by the same cells. This finding parallels the observation that activated NC-37 cells secrete excess free 40-kDa subunit protein (7). The 3′ untranslated sequences of both CLMF subunit mRNAs contain several copies of the octamer motif TTATTTAT (data not shown). This sequence is present in other transiently expressed mRNAs and is involved in regulating mRNA stability (13).

Coexpression of the 40-kDa and 35-kDa CLMF subunit cDNAs in COS cells was required to generate secreted biologically active CLMF (Table 1 and Fig. 3). COS cells transfected with cDNA encoding either the 40-kDa subunit alone or the 35-kDa subunit alone did not secrete biologically active CLMF (Table 1). Mixing media conditioned by COS cells that had been separately transfected with one or the other of the two CLMF subunit cDNAs also did not give rise to bioactive CLMF (Table 1).

Two types of assays were used to compare rCLMF and nCLMF. The first assay measures the proliferation of phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes, whereas the second assay evaluates the synergy between CLMF and suboptimal concentrations of IL-2 in the generation of LAK cells in hydrocortisone-containing cultures (7). The data in Fig. 3 show that rCLMF as expressed in COS cells and nCLMF as purified from NC-37 cells are essentially identical. Dose–response curves for rCLMF and nCLMF were superimposable in each of the two assays, and rCLMF was neutralized by a monoclonal antibody raised against nCLMF. Conditioned media from cultures of mock-transfected COS cells displayed no activity in these assays (Table 1 and data not shown).

DISCUSSION

In a previous report (7), we described the purification of a heterodimeric cytokine, CLMF, that synergized with low amounts of IL-2 to cause the generation of LAK cells in the presence of hydrocortisone and stimulated the proliferation of activated T cells independent of IL-2. In the present report, we have used the N-terminal amino acid sequence information previously obtained to clone the two subunit cDNAs of CLMF. Protein purification of NC-37 cell line-derived CLMF had shown that the protein was composed of two disulfide-bonded subunits with different N-terminal amino acid sequences (7). However, it was not clear from our previous results whether the two subunits were processed from one common gene product and whether proteolytic posttranslational processing other than signal peptide cleavage was occurring. The molecular cloning and sequencing of

Table 1. Coexpression of both CLMF subunit cDNAs is required for secretion of biologically active CLMF by COS cells

Addition	Conc., units/ml	Dilution	[^{3}H]Thymidine incorporated by PHA-activated lymphoblasts, mean cpm ± 1 SEM
Cytokine*			
None	—		11,744 ± 514
nCLMF	200		68,848 ± 878
nCLMF	40		48,827 ± 603
nCLMF	8		26,828 ± 594
nCLMF	1.6		17,941 ± 196
Culture fluid from COS cells transfected with			
A. 35-kDa CLMF subunit cDNA		1:20	11,912 ± 660
		1:100	10,876 ± 232
B. 40-kDa CLMF subunit cDNA		1:20	11,699 ± 931
		1:100	11,666 ± 469
C. 35-kDa + 40-kDa CLMF subunit cDNAs		1:20	58,615 ± 587
		1:100	38,361 ± 828
1:1 mix of culture fluids A and B		1:10†	11,544 ± 483
		1:50	10,503 ± 259
CM from mock-transfected control‡		1:20	11,503 ± 286
		1:100	10,751 ± 303

PHA-activated lymphoblasts were prepared from human peripheral blood mononuclear cells as described (7). Lymphoblast proliferation was measured in a 48-hr assay (7) in which 2×10^4 lymphoblasts were incubated in 100-μl cultures containing the indicated amounts of natural CLMF (nCLMF) or COS cell culture fluids. [^{3}H]Thymidine was added to each culture 18 hr prior to harvest. Conc., concentration.

*nCLMF is purified NC-37-derived CLMF.

†1:10 dilution of the 1:1 mixture of culture fluids A and B was equivalent to a 1:20 final dilution of each of the individual culture fluids.

‡Conditioned medium (CM) from cultures of mock transfected COS cells.

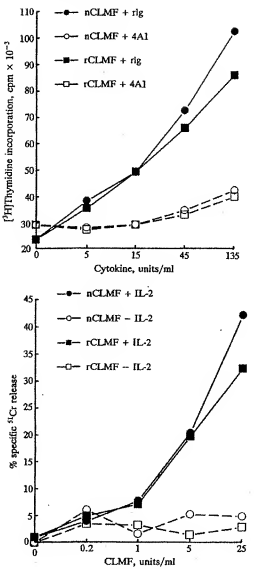

FIG. 3. Comparison of biologic activities of nCLMF (circles) and recombinant CLMF (rCLMF, squares). nCLMF was purified from NC-37 cell-conditioned media; rCLMF was purified from conditioned media from cultures of COS cells transfected with cDNAs encoding the 40-kDa and 35-kDa human CLMF subunits. (*Upper*) T-cell growth factor assay. The ability of CLMF to stimulate the proliferation of human PHA-activated lymphoblasts in 48-hr cultures was assayed as described (7). CLMF preparations were mixed with neutralizing rat monoclonal anti-human CLMF antibody 4A1 (ref. 7; open symbols) or with normal rat IgG (Sigma; rIg, closed symbols) at a final concentration of 20 μg of IgG/ml and were incubated for 30 min at 37°C prior to addition of PHA blasts. All values are means of triplicate determinations. (*Lower*) LAK cell induction assay. The ability of CLMF, alone or in combination with recombinant IL-2, to induce the generation of LAK cells in 4-day cultures was assessed as described (7). Low-density peripheral blood lymphocytes were incubated in the presence of various amounts of nCLMF or rCLMF with (closed symbols) or without (open symbols) recombinant IL-2 at 7.5 units/ml. Units of CLMF activity were based on previous titrations in the T-cell growth factor assay. Hydrocortisone sodium succinate (Sigma) was included at a concentration of 0.1 mM to minimize triggering of endogenous cytokine cascades. Lysis of ^{51}Cr-labeled Daudi cells was assessed at an effector/target ratio of 5:1. The data shown represent the means of quadruplicate determinations. The spontaneous ^{51}Cr release was 20%.

the corresponding cDNAs now has demonstrated that there is no common precursor for the two CLMF subunits; rather, they are encoded by completely different genes. The predicted and actual amino acid composition for the two subunits are strikingly similar; differences in predicted versus actual molecular weights are accounted for by glycosylation (F. Podlaski, personal communication). Thus, the only major posttranslational proteolytic event that appears to take place in the maturation of the CLMF subunits is signal peptide cleavage.

The kinetics of expression of the individual CLMF subunit mRNAs were examined and compared to the expression of mRNAs for IL-2 and the IL-2 receptor p55. Previously it had been observed that NC-37 cells, like certain murine (14) and marmoset (15) B-cell lines, secreted IL-2 when activated (M.K.G., unpublished results). RNA blots demonstrated that upon activation of NC-37 cells, both CLMF subunit mRNAs were coordinately induced with kinetics similar to the IL-2 receptor (p55) mRNAs. On the other hand, IL-2 mRNA levels peaked much earlier. Similar differences in induction kinetics were also seen at the level of IL-2 and CLMF bioactivity secreted from NC-37 cells (M.K.G., unpublished data). These kinetic differences are consistent with our previous observation that in a cytolytic lymphocyte response, IL-2 appears to act earlier than CLMF (5).

Transfection studies with COS cells established that only coexpression of both subunit cDNAs gives rise to secreted bioactive CLMF. Thus, it appears that the two proteins have to interact within the endoplasmic reticulum to assemble properly into bioactive secreted CLMF. By comparing the activity of rCLMF to that of nCLMF in the T-cell growth factor and LAK cell induction assays (Fig. 3) and assuming that the specific activity of rCLMF is similar to that of nCLMF [8×10^7 units/mg (7)], we estimate that the amount of rCLMF heterodimer produced in these experiments was 5–50 ng/ml. The finding that COS cells, which are fibroblast-like cells, are able to assemble correctly the two CLMF subunits to form bioactive CLMF indicates that this secretion and processing pattern is not limited to cells of the lymphoid lineage.

Western blot analysis using an anti-CLMF antibody specific for the 40-kDa subunit has allowed confirmation that (*i*) COS cells transfected with both CLMF subunit cDNAs secrete CLMF with the expected heterodimeric structure and (*ii*) COS cells transfected with the 40-kDa subunit cDNA alone secrete that subunit (F. Podlaski, personal communication). Since no bioactivity was detected in media conditioned by COS cells transfected with only the 40-kDa subunit, that subunit by itself appears either to have a much reduced specific activity compared with heterodimeric CLMF or to be completely inactive.

Because of the lack of a high-affinity antibody specific for the 35-kDa subunit, we have not yet been able to determine definitively whether COS cells transfected with only the 35-kDa subunit cDNA secrete that subunit. Since no bioactivity was detected in the media, secretion of a bioactive 35-kDa subunit by itself could be very inefficient; alternatively, similar to the 40-kDa subunit, the protein could be much less active or inactive altogether. Intracellular 35-kDa protein in the absence of the other subunit could be inherently unstable; there is precedence for this phenomenon, since it has been reported that 90% of the β chains of lutropin (LH), when expressed in the absence of α chains, are retained in the endoplasmic reticulum and are slowly degraded (16). Simple mixing of media conditioned by COS cells transfected separately with either one of the two CLMF subunit cDNAs did not yield bioactive CLMF. One possible explanation would be that the cells do not secrete the 35-kDa CLMF subunit by itself. More likely, our experimental conditions did not allow proper heterodimer formation. One would expect that only

carefully controlled renaturation and oxidation conditions would allow the disulfide bond formation required for generation of bioactive CLMF.

Normal human peripheral blood lymphocytes under the appropriate induction conditions produce both CLMF subunit mRNAs and secrete the active protein (N.N. and M.K.G., unpublished data). There is some evidence suggesting that CLMF is produced predominantly by B cells. In preliminary experiments, B-cell mitogens have appeared to be more effective than T-cell mitogens in eliciting CLMF production from peripheral blood lymphocytes (M.K.G., unpublished results). When screening human cell lines for their ability to produce CLMF activity (7), we observed that four of eight B-cell lines tested produced CLMF after activation with PMA and calcium ionophore, whereas none of five T-cell lines produced CLMF. Nevertheless, three of these T-cell lines secreted large amounts of IL-2 and tumor necrosis factor activity after activation (M.K.G., unpublished results). Likewise, natural killer cell stimulatory factor (NKSF), a heterodimeric cytokine similar or identical to CLMF, was isolated from RPMI 8866 lymphoblastoid B cells (17). A recent report (18) has indicated that B lymphocytes can secrete a cytokine(s) distinct from IL-2 that facilitates virus-specific cytolytic T-lymphocyte responses. It is possible that CLMF may have been the cytokine active in those studies. Thus, although B lymphocytes have not traditionally been viewed as cytokine-producing helper cells, it is conceivable that CLMF production constitutes a novel mechanism whereby B lymphocytes contribute to the amplification of T-lymphocyte responses. In addition to the biologic activities described in this report, CLMF by itself has been shown (*i*) to activate NK cells in an 18–22 hr assay, (*ii*) to facilitate the generation of specific allogeneic CTL responses, and (*iii*) to stimulate the secretion of γ interferon by resting peripheral blood lymphocytes (M.K.G., unpublished results). It can also synergize with low concentrations of recombinant IL-2 in the latter two assays and in causing the proliferation of resting peripheral blood lymphocytes. In view of its production by peripheral blood lymphocytes and its diverse actions on lymphoid cells, it appears that CLMF constitutes a new interleukin. We propose that CLMF be given the provisional designation IL-12. The availability of recombinant CLMF will now make possible a broader and more detailed characterization of its biology.

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EXHIBIT D

Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma

By Amy C. Peterson, Helena Harlin, and Thomas F. Gajewski

Purpose: **Preclinical studies showed that immunization with peripheral blood mononuclear cells (PBMC) loaded with tumor antigen peptides plus interleukin-12 (IL-12) induced CD8$^+$ T-cell responses and tumor rejection. We recently determined that recombinant human (rh) IL-12 at 30 to 100 ng/kg is effective as a vaccine adjuvant in patients. A phase II study of immunization with Melan-A peptide-pulsed PBMC + rhIL-12 was conducted in 20 patients with advanced melanoma.**

Patients and Methods: **Patients were HLA-A2–positive and had documented Melan-A expression. Immunization was performed every 3 weeks with clinical re-evaluation every three cycles. Immune responses were measured by ELISpot assay before and after treatment and through the first three cycles, and were correlated with clinical outcome.**

Results: **Most patients had received prior therapy and had visceral metastases. Nonetheless, two patients achieved a complete response, five patients achieved a minor or mixed response, and four patients had stable disease. The median survival was 12.25 months for all patients and was not yet reached for those with a normal lactate dehydrogenase. There were no grade 3 or 4 toxicities. Measurement of specific CD8$^+$ T-cell responses by direct ex vivo ELISpot revealed a significant increase in interferon gamma–producing T cells against Melan-A (P = .015) after vaccination, but not against an Epstein-Barr virus control peptide (P = .86). There was a correlation between the magnitude of the increase in Melan-A–specific cells and clinical response (P = .046).**

Conclusion: **This immunization approach may be more straightforward than dendritic cell strategies and seems to have clinical activity that can be correlated to a biologic end point.**

J Clin Oncol 21:2342-2348. © 2003 by American Society of Clinical Oncology.

MOST MELANOMA tumors express antigens that can be recognized by CD8$^+$ T cells.[1,2] Nonetheless, tumors frequently escape immune destruction, either from a failure to generate an optimal tumor antigen-specific T-cell response or from development of resistance to the T-cell response induced. One strategy to overcome the former hurdle is through active immunization, the opportunity for which has been facilitated by the molecular definition of melanoma antigens.[3] Specific CD8$^+$ T cells that are properly activated can home to tumor sites and kill tumor cells, to the extent to which they can overcome negative immunoregulatory pathways and tumor resistance.[4]

The optimal immunization strategy for inducing tumor antigen-specific CD8$^+$ effector T cells in humans remains undefined. However, antigen-presenting cell-based strategies have shown promise. Both monocyte-derived[5,6] and bone marrow–derived[7] dendritic cells (DCs) have been loaded with melanoma tumor antigens and administered in the advanced-disease setting, with evidence for immunization and tumor regression in subsets of patients. However, DCs are cumbersome to generate and alternative approaches that are more straightforward yet equally as effective would be useful. One cofactor produced by DCs that contributes to their efficacy is interleukin-12 (IL-12), which facilitates the induction of interferon gamma (IFN-γ)–producing cytolytic effector cells.[8-10] Endogenous IL-12 seems necessary for optimal rejection of immunogenic murine tumors[11,12] and provision of exogenous IL-12, either alone[13] or combined with tumor antigen-based vaccines,[14-19] can induce rejection of pre-established tumors in murine models. We previously have shown that coadministration of IL-12 with peripheral blood mononuclear cells (PBMCs) loaded with tumor antigen peptides induced specific cytolytic T-lymphocyte responses and tumor protection in mice, circumventing the need to generate dendritic cells.[20] The ease by which PBMC can be isolated from patients has made this an attractive approach for clinical translation. We recently conducted a phase I clinical study to determine the dose of recombinant human (rh) IL-12 necessary to induce T-cell responses in combination with antigen-loaded PBMCs, and found that doses from 30 to 100 ng/kg administered subcutaneously (sc) at the vaccine site were optimal and well tolerated.[21] The effective range of doses indicated that a straight dose of 4 μg might be used.

In this article, we describe results of a phase II clinical study of immunization with Melan-A/MART-1[3] peptide-pulsed autologous PBMCs + rhIL-12 in HLA-A2–positive patients with

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Submitted December 26, 2002; accepted March 26, 2003.

Supported by the Burroughs Wellcome Fund, Research Triangle Park, NC, and the Cancer Research Institute, New York, NY.

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0732-183X/03/2112-2342/$20.00

advanced melanoma. Immune responses were analyzed using a direct ex vivo ELISpot assay. We show that this vaccine approach had clinical activity and that the magnitude of increased T-cell response correlated with clinical outcome.

PATIENTS AND METHODS

Patient Enrollment and Eligibility

This was an open-label, nonrandomized, single-institution study of Melan-A peptide-pulsed autologous PBMCs + rhIL-12.[4] The protocol was approved by the University of Chicago Institutional Review Board and all patients signed written informed consent. Patients who were both HLA-A2–positive and showed Melan-A tumor expression by reverse transcriptase polymerase chain reaction (RT-PCR) were considered for inclusion. Additional inclusion criteria were life expectancy more than 12 weeks, Karnofsky performance status ≥70, and adequate hematopoietic, renal, and hepatic function. Delayed-type hypersensitivity (DTH) skin testing was performed against mumps, *Candida*, and *Trichophyin*, not for eligibility but to correlate subsequently with clinical outcome and immunization potential. Patients were excluded if they had severe cardiovascular disease or arrhythmia, were pregnant or nursing, had biologic therapy received within 4 weeks, tested positive for hepatitis B surface antigen or human immunodeficiency virus (HIV), had clinically significant autoimmune disease or any illness requiring immunosuppressive therapy, had a psychiatric illness that would interfere with patient compliance and informed consent, had active gastrointestinal bleeding or uncontrolled peptic ulcer disease, or had uncontrolled brain metastases. Patients with treated brain metastases who were clinically and radiographically stable and did not require corticosteroids were allowed to enter onto the trial.

Patient Characteristics

Twenty patients with metastatic melanoma were enrolled after giving written informed consent. Patient characteristics are outlined in Table 1. All patients had advanced disease; the majority had at least three sites of metastasis, 60% of which were visceral (ie, noncutaneous and nonpulmonary metastases). Approximately two thirds of the patients had received prior therapy, and 10 patients had an elevated lactate dehydrogenase (LDH) level, which is an important negative prognostic factor.[22] Only 45% were positive for at least one recall antigen (mumps, *Candida*, or *Trichophytin*) by DTH skin testing.

Table 1. Patient Characteristics

Patient Characteristic	Patients (n = 20) No.	%*
Age, years		
Median	58	
Range	35-79	
Sex		
Male	9	45
Female	11	55
Karnofsky performance status (ECOG)		
90%-100% (0)	10	50
70%-80% (1)	9	45
60%-70% (2)	1	5
No. of metastatic sites		
1	2	10
2	None	
≥3	18	90
Location of metastases		
Visceral	13	65
Brain (treated)	4	20
Prior therapy		
None	6	30
Chemotherapy or immunotherapy	7	35
As only prior therapy	5	25
Chemotherapy	1	5
As only prior therapy	1	5
Immunotherapy	4	20
As only prior therapy	1	5
Other*	2	10
As only prior therapy	None	
Adjuvant IFN-α	5	25
As only prior therapy	3	15
Elevated LDH	10	50
DTH recall positive	9	45

Abbreviations: ECOG, Eastern Cooperative Oncology Group; IFN-α, interferon alfa-2b; LDH, lactate dehydrogenase; DTH, delayed-type hypersensitivity.

*Experimental therapy other than a melanoma vaccine, immunomodulatory cytokine, or chemotherapy.

RT-PCR Analysis

RNA was isolated from fresh tumor cells using guanidine and cesium chloride. cDNA was synthesized and PCR was performed for Melan-A and beta-actin using the primer pairs and reaction conditions described previously.[21] Control reactions without reverse transcriptase were performed to rule out a contribution of genomic DNA. PCR products were visualized using a 1.5% ethidium bromide–stained agarose gel. No formal quantitation was performed.

Vaccine Preparation

Therapy consisted initially of three 21-day cycles. Vaccinations were given on the first day of each cycle and rhIL-12 was administered subcutaneously on days 1, 3, and 5. Approximately 100 to 150 mL of peripheral blood from patients was collected on day 1 of each cycle into heparinized 60-mL syringes using sterile technique. PBMCs were isolated over a Lymphoprep gradient (Lymphoprep; Axis-Shield PoC, Oslo, Norway), counted, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) at 40×10^6 cells/mL. At least 10×10^6 cells from each sample were cryopreserved to prepare $CD8^+$ and $CD8^-$ fractions for subsequent correlative immunologic studies. The Melan-A_{27-35} peptide (AAGIGILTV) was produced according to good manufacturing practice standards by Multiple Peptide Systems (San Diego, CA) and provided in lyophilized vials. Aliquots of peptide were prepared at 5 mmol/L in dimethyl sulfoxide and stored at −80°C for up to 3 months. Peptide preparations were quality controlled for HLA-A2 binding, sterility, and identity by high-performance liquid chromatography and mass spectrometry. An aliquot of peptide was diluted to 20 mmol/L in DPBS and mixed with an equal volume of patient PBMCs (final peptide concentration, 10 μmol/L; target number of PBMCs, 10^8) followed by incubation at 37°C for 1 hour in 10 mL DPBS. The cells were then irradiated (20 Gy), washed in DPBS, and resuspended in 1 mL DPBS. The suspension of peptide-loaded PBMCs was injected sc using a 1-mL syringe and a 21-gauge needle, divided evenly into two sites. Preferred sites were those near draining lymph node basins but not near a tumor mass. The actual number of PBMCs administered per vaccine ranged from 78 to 100×10^6.

rhIL-12 was provided by Genetics Institute (Cambridge, MA) as a lyophilized powder of 10 μg under vacuum. Each vial was intended for single use only and was stored as a powder in our research pharmacy at 2 to 8°C until reconstituted with sterile water for injection. Once reconstituted, rhIL-12 was loaded into 3-mL syringes and used within 4 hours. rhIL-12 (4 μg) was administered sc with a 25-gauge needle just after pulsed PBMC inoculation and immediately adjacent to one of the two immunization sites on days 1, 3, and 5. The same approximate location was used for each injection of peptide-pulsed PBMCs and rhIL-12 for each cycle.

Toxicity Assessment and Criteria for Clinical Response

Toxicities were determined using the National Cancer Institute common toxicity criteria scale version 2.0. A complete response (CR) was assigned if there was disappearance of all lesions without the appearance of any new

lesions; a partial response (PR) was defined as ≥ 50% reduction in total tumor volume; a minor response (MR) was defined as less than 50% reduction in total tumor volume; progressive disease (PD) was assigned if new lesions appeared, any tumor reappeared, or if a 25% increase in tumor area was observed; a mixed response was assigned if at least one tumor decreased in size with other or new tumors growing; stable disease (SD) was anything that did not fit the aforementioned criteria. When possible, cutaneous lesions were photographed.

$CD8^+$ T-Cell Preparation

$CD8^+$ and $CD8^-$ fractions from PBMC were isolated at the time of preparation of each vaccine and cryopreserved until analysis in batch fashion. $CD8^+$ T lymphocytes were isolated by positive selection using CD8 microbeads and magnetic columns (MACS system; Miltenyi Biotech, Auburn, CA). The unbound $CD8^-$ fraction was cryopreserved for use as antigen-presenting cells for in vitro expansion of specific $CD8^+$ T cells. Although the primary ELISpot analysis was performed directly with thawed cells, a secondary assay was carried out after in vitro expansion. For in vitro expansion, $CD8^-$ cells were thawed from each time point and pooled, pulsed with 50 μmol/L Melan-A peptide in serum-free Iscove's modified Dulbecco's medium (IMDM) with beta$_2$-microglobulin, irradiated (3,000 rad), washed, and plated at 2×10^6 cells/well in 24-well plates. $CD8^+$ T cells were thawed and cultured with the irradiated $CD8^-$ cells at 4×10^5 cells/well in IMDM medium containing 10% human AB serum. After 5 days, the cells were collected and plated with a new batch of Melan-A–pulsed irradiated $CD8^-$ cells. After an additional 5 days the cells were collected and tested.

ELISpot Assays

Briefly, 96-well membrane bottomed plates (MAHA S4510; Millipore, Bedford, MA) were coated with 15 μg/mL of antihuman IFN-γ antibody (Mabtech, Cincinnati, OH) in PBS. The plates were washed and $CD8^+$ T cells, either freshly thawed at 5×10^4 cells/well or after in vitro expansion at 5×10^3 cells/well, were plated in triplicate in IMDM medium with 10% human AB serum. T2 cells (transporter associated with antigen processing–deficient cell line, American Type Culture Collection no. CRI 1992) were pulsed for 1 hour at 37°C with 50 μmol/L peptide (either derived from HIV [ILKEPVHGV], Epstein-Barr virus [EBV; GLCTLVAML], or Melan-A [AAGIGILTV]), washed, and plated at a 5-to-1 ratio to the T cells. A replicate of $CD8^+$ T cells was stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) + ionomycin (0.5 μg/mL) as a positive control. After 24 hours, the cells were removed by washing with PBS + 0.05% Tween (wash buffer), and biotinylated antihuman IFN-γ antibody was added in PBS + 0.5% fetal calf serum. The plates were incubated for 2 to 4 hours at room temperature, washed, and streptavidin–alkaline phosphatase was added for 1 hour at room temperature. The plates were then washed, BCIP-NBT (5-bromo–4-chloro-3-indolyl phosphate/nitro-blue tetrazolium) was added, and the plates were finally washed with water and allowed to air dry. Plates were scanned with an ELISpot reader (CTL Technologies, Cleveland, OH) and the number of spots per well was enumerated after the background was set on the basis of wells that had been incubated with medium alone; spot separation was adjusted using Immunospot software (CTL Technologies). For each sample, the number of T cells producing IFN-γ in response to EBV or Melan-A peptides was determined by subtracting the number of spots seen in response to HIV peptide. The mean and SD were determined for each triplicate sample. After immunization, the time point at which peak frequencies among the first three cycles were observed was used for data analysis.

Statistical Analysis

Comparisons between pre- and post-ELISpot frequencies were performed using a paired *t* test, and comparisons of augmented ELISpot frequencies between responders and nonresponders were made using an unpaired two-sided *t* test. Correlations between various dichotomous variables and clinical outcome were made using Fisher's exact test (two-sided). Survival data were determined using the Kaplan-Meier method, with differences among subgroups assessed by the log-rank test. All analyses were performed using SPSS software (version 8.0; SPSS Inc, Chicago, IL).

Table 2. Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3
Fatigue	16	0	0
Anorexia	6	0	0
Fever	7	0	0
Rash	3	0	0
Headache	3	0	0
Nausea	2	0	0
Injection site reaction	5	0	0
Neutropenia	1	2	0
Thrombocytopenia	2	0	0
Hepatic	5	2	0
Creatinine	1	0	0

NOTE. Adverse events were determined using the National Cancer Institute common toxicity criteria scale version 2.0.

RESULTS

Immunization Treatment and Toxicities

Each 3-week cycle consisted of immunization on day 1 and sc rhIL-12 administration on days 1, 3, and 5, as described in Methods. Three cycles constituted one course of therapy and patients were evaluated for response after each course. Patients were observed as inpatients in our General Clinical Research Center for the first 24 hours of each cycle.

Adverse reactions are listed in Table 2. All but one patient completed at least three cycles of therapy. There were no grade 3 to 4 toxicities; two patients had grade 2 neutropenia and two patients had grade 2 ALT or AST elevations, which were reversible. The most common adverse reactions were fatigue and fever.

Clinical Outcome

Clinical response outcomes are listed in Table 3. Two patients had a CR, for an overall response rate of 10%. In addition, four patients (20%) had a mixed response, one patient (5%) had an MR, four patients (20%) had SD, and the remaining nine patients (45%) had PD. The sites of tumor response were diverse. The two patients who experienced a CR both had numerous metastases of 2 cm or less and a normal LDH. One patient was female, had multiple cutaneous lesions, and no prior therapy; the other patient was male, had multiple lung lesions, and had experienced prior treatment failure from chemoimmunotherapy. Neither patient experienced a recurrence with a mean follow-up time of 28 months at the time of data analysis. Of the five other patients who showed a decrease in size of at least one tumor mass, three had responses in skin, one had a response in bone, and one had a response in an adrenal lesion. Three of the four patients with SD had visceral metastases.

Table 3. Clinical Outcome

Best Response	No. of Patients	%
Complete response	2	10
Partial response	0	0
Minor response	1	5
Mixed response	4	20
Stable disease	4	20
Progressive disease	9	45

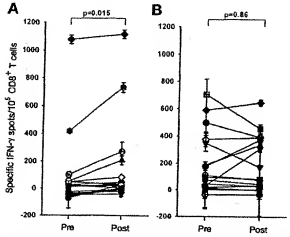

Fig 1. Interferon gamma ELISpot frequencies by $CD8^+$ T cells against Melan-A and (A) Epstein-Barr virus (EBV) (B) pre- and postimmunization. Control values with HIV peptide were subtracted out. Post- and pretreatment values were compared using a paired *t* test.

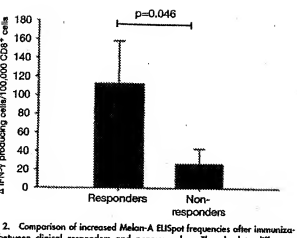

Fig 2. Comparison of increased Melan-A ELISpot frequencies after immunization between clinical responders and nonresponders. The absolute difference between Melan-A-specific ELISpot frequencies post- and pretreatment was compared between responders and nonresponders using a two-sided, unpaired *t* test.

Peptide-Specific T-Cell Responses by ELISpot

A carefully controlled IFN-γ ELISpot assay was used to monitor the immune response to immunization. Cryopreserved $CD8^+$ T cells were thawed in batch fashion and stimulated in triplicate directly ex vivo with T2 cells loaded with peptides derived from either HIV, EBV, or Melan-A. The HIV values were subtracted from those obtained with either Melan-A or EBV as an internal control at each time point. Seventeen of the enrolled patients had adequate cryopreserved material with which to perform immunologic assessments.

As shown in Fig 1, some patients displayed a high frequency of Melan-A–specific $CD8^+$ T cells before vaccination, with as high as 1% of $CD8^+$ cells responding to this peptide. These T cells were functional because they produced IFN-γ. The majority of patients showed an increase in the frequency of Melan-A–specific cells after immunization ($P = .015$). In contrast, the frequencies of specific $CD8^+$ T cells responding to the EBV peptide did not vary significantly overall ($P = .86$). Although the changes in T-cell frequency were modest, these results demonstrate an antigen-specific response after immunization with Melan-A peptide-pulsed PBMC + rhIL-12.

The changes in Melan-A–specific ELISpot frequencies were compared among patients who had a mixed response or better and those who had no clinical response. As shown in Fig 2, the mean increase in Melan-A–specific T cells for the clinical responders was 112 ± 45 and for nonresponders was 26 ± 16, indicating that a greater absolute increase in Melan-A–specific T cells was associated with tumor regression ($P = .046$).

Survival and Associations Between Immunologic Parameters and Clinical Outcome

The overall median survival was 12.25 months and is shown in Fig 3A. Seven patients remained alive at the time of data analysis, with all patients followed beyond 12 months. Because the presence of elevated levels of serum LDH is a known negative prognostic factor,[23] survival was also compared in response to this vaccine on the basis of LDH level (Fig 3B). The median survival for patients with an elevated LDH level was 9.25 months, whereas the median had not yet been reached for those with a normal LDH ($P = .005$). In addition, the median survival for patients who experienced a significant increase in Melan-A–specific T cells was not yet reached, compared with 8.5 months for patients without a significant increase in Melan-A–specific cells (Fig 3C; $P = .120$).

Additional immunologic parameters that had been measured were also analyzed for associations with either clinical response or survival and are summarized in Table 4. Neither a positive recall DTH to standard antigens nor a relatively high number of EBV- or Melan-A–specific $CD8^+$ T cells before immunization correlated with either outcome. The median pretreatment Melan-A–specific T cell frequency was 23 in clinical nonresponders and −26 in responders. To increase the sensitivity of the assay to detect Melan-A–specific T cells, an in vitro expansion was performed on the preimmunization samples and analyzed by ELISpot as described in Methods. Ten patients showed high Melan-A–specific T cell frequencies after in vitro expansion. However, this also failed to correlate with clinical outcome. Finally, although a normal LDH level was associated with survival, it did not correlate with clinical response and also did not correlate with immune response. Collectively, these results reinforce the specificity of the result showing a significant association between an increased number of Melan-A–specific T cells and clinical outcome.

Expression of Melan-A in Resected Tumors After Immunization

It was conceivable that some patients developed PD despite immunization because of outgrowth of Melan-A–negative tumor cells. Posttreatment tumor samples were obtained from progressing tumors from three patients and analyzed by RT-PCR. Although the new metastasis that developed in patient 1 was negative for

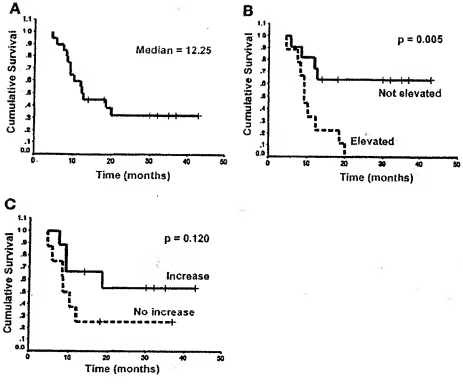

Fig 3. Overall survival for all patients (A), on the basis of serum lactate dehydrogenase greater than 200 U/L (B), and on the basis of increased Melan-A-specific interferon gamma-producing $CD8^+$ T cells (C) was determined using the Kaplan-Meier method. Differences between groups were compared using the log-rank test.

Melan-A expression, those samples from patients 4 and 6 retained detectable expression of Melan-A mRNA (Fig 1). These results indicate that, although outgrowth of antigen-negative tumors can occur, other mechanisms of resistance to immune destruction likely explain the lack of clinical response in other patients.

DISCUSSION

In this study we used Melan-A peptide-pulsed autologous PBMC + rhIL-12 as a vaccine to treat HLA-A2–positive patients with advanced melanoma. We observed a significant increase in Melan-A–specific IFN-γ–producing $CD8^+$ T cells after immunization, and found a statistical association between clinical response and the magnitude of the specific T-cell increase. Although it is difficult to compare across individual, small phase II studies, these results are similar to those that have been reported using antigen-loaded dendritic cells, but with a strategy that may be more straightforward to execute.

Preparation of the peptide-loaded PBMCs typically took 5 hours from phlebotomy to injection, and quality control of the cell product was facilitated by the lack of an extended in vitro culture period and absence of exposure to culture medium or serum proteins that is required for dendritic cell preparations. Conversely, dendritic cell vaccines have been prepared in batches and cryopreserved in individual doses in some studies, which obviates the need to prepare a fresh vaccine at each time point. Cryopreservation of vaccines has not yet been examined with our current approach. A comparative trial between PBMC/rhIL-12 and dendritic cell-based vaccination may, therefore, be of interest as the technologies continue to develop. Our results support the notion developed in preclinical models that IL-12 can contribute to effective antitumor immunity, and are consistent with the results of a recent adjuvant vaccine study using rhIL-12 in melanoma.[24]

We used a direct ex vivo ELISpot assay to assess antigen-specific T-cell responses in this study. Control experiments testing EBV reactivity from normal donors revealed that ELISpot analysis could be performed accurately on cryopreserved $CD8^+$ T cell samples immediately after thawing (H. Harlin and T. Gajewski, unpublished data). We found that background reactivity against the control HIV peptide varied among patients and to some extent among time points for an individual patient. The magnitude of increase in apparent Melan-A–reactive T cells would have been greater in some patients had the values obtained with the HIV control peptide not been subtracted. We believe that this experimental detail is critical because it normalizes the samples for background differences and provides an internal control for minor variation between individual vials of cryopreserved T cells. We also compared the Melan-A frequencies to those against an EBV control peptide, to determine whether the treatment was altering ELISpot results. We performed our analyses on purified $CD8^+$ T cells to control for variable numbers between patients and across time points. It is possible that we excluded subpopulations of $CD8^-$ T cells, $CD4^+$ T cells, and natural killer T cells that could have produced IFN-γ in response to Melan-A. Nonetheless, our results revealed a measurable and significant increase in Melan-A–specific T cells posttreatment. Our currently employed ELISpot assay is distinct from the assay used in our phase I trial of peptide-pulsed

Table 4. Statistical Correlates With Response or Survival

Parameter	Correlation With Response (P)	Correlation With Survival (P)
Positive DTH recall	.642	.130
Strong EBV pre-Rx*	.131	.491
Increased EBV post versus pre†	.290	.644
Strong Melan-A pre-Rx†	.644	.481
Increased Melan-A post versus pre†	.046	.120
Strong in vitro expansion of Melan-A§	.304	.565
LDH levels < 200	.99	.005

NOTE. Associations with response were determined using Fisher's exact test (two sided), except the differences between pre- and posttreatment, which were determined using an unpaired *t* test. Associations with survival were determined using the Kaplan-Meier method and log-rank test. Significant values are indicated in boldface.

Abbreviations: DTH, delayed-type hypersensitivity; EBV, Epstein-Barr virus; Rx, immunization; LDH, lactate dehydrogenase; HIV, human immunodeficiency virus; IL-2, interleukin-2.

*At least 90 spots per 10^5 $CD8^+$ T cells after subtraction of background against a control HIV peptide.

†Changes between post- and prevaccination samples were calculated as the difference between the absolute number of specific spots and compared using an unpaired *t* test between clinical responders and nonresponders.

‡At least 40 spots per 10^5 $CD8^+$ T cells after subtraction of background against a control HIV peptide.

§At least 90 spots per 10^5 $CD8^+$ T cells after subtraction of background against a control HIV peptide, after a 10-day in vitro expansion with Melan-A peptide-pulsed autologous $CD8^-$ cells and IL-2.

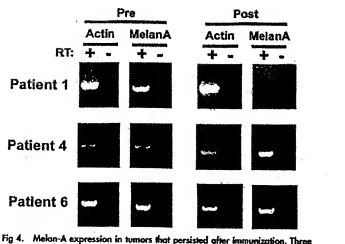

Fig 4. Melan-A expression in tumors that persisted after immunization. Three patients underwent surgical resection of lesions (after discontinuing the study), which were analyzed for Melan-A expression by qualitative reverse transcriptase polymerase chain reaction. Controls were analyzed without reverse transcriptase or with beta-actin primers.

PBMC + rhIL-12 and in other trials[21,25] in which in vitro expansion had been performed before assessment of IFN-γ production. Analysis of T-cell responses with minimal in vitro manipulation should most accurately reflect the status of those cells in vivo.

High frequencies of Melan-A–specific, IFN-γ–producing $CD8^+$ T cells were observed in some patients at study entry when they clearly had progressively growing melanoma. This observation indicates that the absolute frequency of functional T cells against a tumor antigen does not correlate with the behavior of the tumor. We also found no statistical association between this high frequency and clinical outcome; in fact, the two patients who experienced a CR had undetectable Melan-A–specific T cells before therapy. Although high frequencies of T cells reacting with a Melan-A tetramer have been detected in some normal donors,[26] those cells had a naïve surface phenotype and did not produce high levels of IFN-γ. What did correlate with clinical response in our current study is a meaningful increase in Melan-A–specific T cells posttreatment. These increases were modest (a net gain of 112 spots per 10^5 $CD8^+$ T cells on average), indicating either that a subtle alteration in the steady-state between the immune response and a growing tumor in favor of increased T-cell frequencies is sufficient to translate into tumor regression, or that another immune function that we are not measuring is contributing to the final event of tumor shrinkage. Tumor regressions without detectable increases in T-cell frequencies using standard assays have been observed in other studies.[27]

The median overall survival in our study was 12.25 months from treatment initiation, which is greater than the expected 6 to 9 months for this patient population. Although it was a relatively small study and subject to selection bias, most patients were pretreated and had visceral disease, one half of the patients had elevated serum LDH levels, and four patients had treated brain metastases. As has been seen in melanoma patients treated with standard therapies, we found that an elevated serum LDH level was a negative prognostic factor for survival. Whether this is reflective of tumor burden or the metabolic state of the tumor cells that have adapted to an anaerobic environment is unclear.

Some patients developed increases in Melan-A–specific T cells and developed progressive tumor growth despite retained expression of the antigen on posttreatment biopsies. This observation is similar to that seen in murine studies[28] and indicates mechanisms of tumor resistance downstream from initial T-cell priming, presumably within the tumor microenvironment. Potential explanations include poor T-cell trafficking to tumor sites, presence of negative regulatory cells, T-cell anergy or death, expression of inhibitory molecules by tumor cells, or downregulation of class I major histocompatibility complex or antigen-processing molecules.[29,30] Future studies should investigate definable mechanisms of tumor escape that allow tumor cells to resist elimination by antigen-specific T cells in vivo.

ACKNOWLEDGMENT

We thank Genetics Institute/Wyeth for rhIL-12, and T. Kerrison, M. Sherman, S. Swiger, and M. Posner for important contributions.

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EXHIBIT E

Vaccination with Mage-3A1 Peptide–pulsed Mature, Monocyte-derived Dendritic Cells Expands Specific Cytotoxic T Cells and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

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Summary

Dendritic cells (DCs) are considered to be promising adjuvants for inducing immunity to cancer. We used mature, monocyte-derived DCs to elicit resistance to malignant melanoma. The DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. 11 far advanced stage IV melanoma patients, who were progressive despite standard chemotherapy, received five DC vaccinations at 14-d intervals. The first three vaccinations were administered into the skin, 3×10^6 DCs each subcutaneously and intradermally, followed by two intravenous injections of 6×10^6 and 12×10^6 DCs, respectively. Only minor (less than or equal to grade II) side effects were observed. Immunity to the recall antigen was boosted. Significant expansions of Mage-3A1–specific $CD8^+$ cytotoxic T lymphocyte (CTL) precursors were induced in 8/11 patients. Curiously, these immune responses often declined after the intravenous vaccinations. Regressions of individual metastases (skin, lymph node, lung, and liver) were evident in 6/11 patients. Resolution of skin metastases in two of the patients was accompanied by erythema and $CD8^+$ T cell infiltration, whereas nonregressing lesions lacked $CD8^+$ T cells as well as Mage-3 mRNA expression. This study proves the principle that DC "vaccines" can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active $CD8^+$ CTL–tumor cell interaction in situ as well as escape by lack of tumor antigen expression.

Key words: dendritic cells • vaccination • active immunotherapy • melanoma • cytotoxic T lymphocytes

It is now established that the immune system has cells, particularly $CD8^+$ CTLs, that can recognize tumor antigens and kill tumors (1, 2). Nevertheless, a major problem is that these T cells are either not induced or only weakly induced, i.e., the T cells are not evident in the systemic circulation. One possibility is that there is inadequate tumor antigen presentation by dendritic cells (DCs),[1] "nature's adjuvant" for eliciting T cell immunity (3). Another is that tumor-reactive T cells are tolerized by the tumors (1, 4). Melanoma provides a compelling setting in which to pursue a current goal of cancer immunotherapy, the generation of stronger tumor-specific T cell immunity, particularly with CTLs (4). The majority of tumor antigens identified so far are expressed by melanomas (2). Limited antimelanoma CTL responses have been detected (5), and infusions of IL-2 expanded killer cells can lead to rejection of melanoma (6).

Conventional adjuvants promote antibody rather than CTL responses. Therefore, several novel strategies are being explored to induce tumor-specific T cell immunity. DC vaccination is one of these (3). Immature DCs capture

[1] *Abbreviations used in this paper:* CNS, central nervous system; DCs, dendritic cells; DTH, delayed-type hypersensitivity; MCM, monocyte-conditioned medium; RT, reverse transcriptase; TT, tetanus toxoid.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/12/1669/10 $5.00
Volume 190, Number 11. December 6, 1999 1669–1678
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antigens but lack full T cell–stimulatory activity (7). In the presence of appropriate stimuli, such as inflammatory cytokines, the DCs mature. DCs upregulate T cell adhesion and costimulatory molecules as well as select chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. The use of DCs as adjuvants is supported by many animal experiments with primarily mature DCs (3, 8). These studies have shown that the injection of tumor antigen–loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases (3, 8). In the few pilot trials reported so far for humans, *immature* DCs have been employed (9–11). Scattered tumor responses are reported, but evidence for the induction of tumor-specific CTLs by DC vaccination has not been shown.

We have developed a technique to generate large numbers of homogenous populations of *mature* and stable DCs from monocytes in the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we provide the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1 peptide–pulsed mature DCs reliably enhance Mage-3A1–specific $CD8^+$ and recall $CD4^+$ T cell immunity in heavily pretreated, progressive stage IV melanoma patients with large tumor loads. Expansions of Mage-3A1–specific CTL responses have not been previously detected after Mage-3A1 peptide vaccination in less advanced melanoma patients (14), underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon DC-mediated immunization and were accompanied by $CD8^+$ T cell infiltration, we propose that the induced Mage-3A1–specific CTLs are active in vivo.

Materials and Methods

Patient Eligibility Criteria

Patients were eligible if they suffered from stage IV (i.e., distant metastases) cutaneous malignant melanoma (1988 American Joint Committee on Cancer/Union Internationale Contre Cancer pTNM staging system) that was not curable by resection and was progressive despite chemo(immuno)therapy. Further inclusion criteria were an expected survival ≥4 mo, Karnofsky index ≥60%, age ≥18 yr, measurable disease, HLA-A1 positivity, expression of Mage-3 gene shown by reverse transcriptase (RT)-PCR in at least one excised metastasis, and no systemic chemo-, radio-, or immunotherapy within 4 wk (6 wk in the case of nitrosurea drugs) preceding the first DC vaccination. A positive skin test to recall antigens was *not* required. Important exclusion criteria were active central nervous system (CNS) metastasis, any significant psychiatric abnormality, severely impaired organ function (hematological, renal, liver), active autoimmune disease (except vitiligo), previous splenectomy or radiation therapy to the spleen, organ allografts, evidence for another active malignant neoplasm, pregnancy, lactation, or participation (or intent to participate) in any other clinical trial. Concomitant treatment (chemo- or immunotherapy, corticosteroids, investigational drugs, paramedical substances) was prohibited. Palliative radiation or surgical therapy of selected metastases and certain medications (acetaminophen/paracetamol, nonsteroidal anti-inflammatory drugs, opiates) to control symptoms were allowed.

Clinical Protocol and Study Design

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz, Germany according to standards of Good Clinical Practice for Trials on Medicinal Products in the European Community. The protocol was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (New York, NY) and performed under supervision of its Office of Clinical Trials Management as study LUD #97-001. The protocol was also approved by the ethics committees of the involved study centers.

The study design is shown in Table II. All patients gave written informed consent before undergoing a screening evaluation to determine their eligibility. Extensive clinical and laboratory assessments were conducted at visits I, 5, and 8 (Table II) and consisted of a complete physical examination, staging procedures, and standard laboratory values as well as special ones (pregnancy test, free testosterone in males, autoantibody profile, and antibodies to HIV-1/2, human T cell lymphotropic virus type I, hepatitis B virus, and hepatitis C virus). Patients were hospitalized and examined the day before each vaccination and were monitored for 48 h after the DC injections. Adverse events and changes in laboratory values were graded on a scale derived from the Common Toxicity Criteria of the National Cancer Institute, National Institutes of Health, Bethesda, MD.

Production of the DC Vaccine

During prestudy screening, we tested a small amount of fresh blood to verify that appropriate numbers of mature DCs could be generated from the patient's monocytes (12). Sufficient DC numbers could be successfully generated in all patients, but in some patients the test generation revealed that TNF-α had to be added to assure full maturation. To avoid repetitive blood drawings, we performed a single leukapheresis during visit 2 to generate DCs as described (13). In short, PBMCs from the leukapheresis ($\geq 10^{10}$ nucleated cells) were isolated on Lymphoprep™ (Nycomed Pharma) and divided into three fractions. The first fraction of 10^9 PBMCs was cultured on bacteriological petri dishes (Cat. #1005; Falcon Labware) coated with human Ig (100 μg/ml; Sandoglobin™; Sandoz GmbH) in complete RPMI 1640 medium (BioWhittaker) supplemented with 20 μg/ml gentamicin (Refobacin 10; Merck), 2 mM glutamine (BioWhittaker), and 1% heat-inactivated human plasma for 24 h to generate monocyte-conditioned medium (MCM) for later use as the DC maturation stimulus. The second fraction of 3×10^8 PBMCs was used for the generation of DCs for vaccination 1 and delayed-type hypersensitivity (DTH) test I. Adherent monocytes were cultured in 1,000 U/ml GM-CSF (10×10^7 U/mg; Leukomax™; Novartis) and 800 U/ml IL-4 (purity >98%; 4.1×10^7 U/mg in a bioassay using proliferation of human IL-4R$^+$ CTLL; CellGenix; expressed in *Escherichia coli* and produced under good laboratory practice conditions but verified for good-manufacturing practice [GMP] safety and purity criteria by us) for 6 d, and then MCM was added to mature the DCs. MCM was supplemented in patients 04, 06, 09, 11, and 12 with 10 ng/ml GMP-rhu TNF-α (purity >99%; 5×10^7 U/mg in a bioassay using murine L-M cells; a gift of Dr. G.R. Adolf, Boehringer Ingelheim Austria, Vienna, Austria) to assure full maturation of DCs. Mature DCs were harvested on day 7. The third fraction of PBMCs was frozen in aliquots and stored in the gas phase of liquid nitrogen to generate DCs for later vaccinations and DTH tests.

DCs for vaccinations were pulsed with the Mage-3A1 peptide (15) (EVDPIGHLY, synthesized at GMP quality by Clinalfa) as tumor antigen, and as a recall antigen and positive control, tetanus toxoid (TT) or tuberculin (if at visit 1 the DTH to TT in the

Multitest Merieux was >10 mm; both purchased from the Bacterial Vaccines Department of the Statens Serum Institute, Copenhagen, Denmark). The recall antigen was added at 10 μg/ml for the last 24 h, and the Mage-3A1 peptide was added at 10 μM directly to the cultures for the last 8 h (if immunity to recall antigens was strongly boosted, the dose of recall antigen was reduced to 1.0 or 0.1 μg/ml or was omitted for the intravenous DC injections to avoid a cytokine release syndrome). On day 7, mature DCs were harvested, resuspended in complete medium, washed, and pulsed once more with Mage-3A1 peptide (now at 30 μM) for 60 min at 37°C. DCs were finally washed and resuspended in PBS (GMP quality PBS; BioWhittaker) for injection. DCs to be used for Mage-3A1 DTH tests were pulsed with Mage-3A1 (but no recall antigen); DCs that served as negative control in the DTH tests were not pulsed at all. An aliquot of the DCs to be used for vaccinations was analyzed as described (13) to assure that functionally active and mature DCs were generated. The features of the DCs are described in Results. Release criteria were typical morphology (>95% nonadherent veiled cells) and phenotype (>95% HLA-DR^{+++}CD86^{+++}CD40^{+}CD25^{+}CD1^{-} and >65% homogenously CD83^{++}).

Immunization Schedule

A total of five vaccinations (three into the skin followed by two intravenously) with antigen-pulsed DCs were given at 14-d intervals (Table II). This design was chosen to explore the toxicity and efficacy of various routes in this trial. For vaccinations 1–3, 3×10^6 DCs were given subcutaneously at two sites (1.5×10^6 DCs in 500 μl PBS per site) and 3×10^6 intradermally at 10 sites (3×10^5 DCs in 100 μl PBS per site). The injection sites were the ventromedial regions of the upper arms and the thighs close to the regional lymph nodes and were rotated clockwise. Limbs where draining lymph nodes had been removed and/or irradiated were excluded. For intravenous vaccinations 4 and 5, a total of 6 and 12×10^6 antigen-pulsed DCs (resuspended in 25 or 50 ml PBS plus 1% autologous plasma) was administered over 5 and 10 min, respectively. Premedication with an antipyretic (500 mg acetaminophen/paracetamol p.o.) and an antihistamine (2.68 mg clemastinhydrogenfumarat i.v.) was given 30 min before intravenous DC vaccination.

Evaluation of Immune Status

Recall Antigen–specific Proliferation and Cytokine Production. PBMCs were cultured in triplicate at two dose levels (3×10^4 and 1×10^5 PBMCs/well) plus or minus TT or tuberculin (at 0.1, 1, and 10 μg/ml) and pulsed on day 5 with [^{3}H]thymidine for 12 h. In all cases, the highest cpms were obtained with the highest doses of PBMCs and antigen and are shown in Fig. 2. IL-4 and IFN-γ levels were measured in culture media by ELISA (Endogen, Inc.). In a separate plate, staphylococcal enterotoxin (SEA; Serva) was added at 0.5, 1, and 5 ng/ml, and proliferation was assessed after 3 d to provide a positive control for helper T cell viability and responsiveness.

Enzyme-linked Immunospot Assay for IFN-γ Release from Single Antigen–specific T Cells. To quantitate antigen-specific, IFN-γ–releasing, Mage-3A1–specific effector T cells, an enzyme-linked immunospot (ELISPOT) assay was used as described (16). PBMCs (10^5 and 5×10^5/well) or in some cases CD8$^+$ or CD4$^+$ T cells (isolated by MACS™ according to the manufacturer, Miltenyl Biotec) were added in triplicate to nitrocellulose-bottomed 96-well plates (MAHA S4510; Millipore Corp.) precoated with the primary anti–IFN-γ mAb (1-D1K; Mabtech) in 50 μl ELISPOT medium (RPMI 1640 and 5% heat-inactivated human serum) per well. For the detection of Mage-3A1–reactive T cells, the APCs were irradiated T2.A1 cells (provided by P. van der Bruggen, Ludwig Institute of Cancer Research, Brussels, Belgium) pulsed with MHC class I–restricted peptides (Mage-3A1 peptide and the HIV-1 p17-derived negative control peptide GSEELRSLY) added at 7.5×10^4/well (final volume 100 μl/well). After incubation for 20 h, wells were washed six times, incubated with biothylated second mAb to IFN-γ (7-B6-1; Mabtech) for 2 h, washed, and stained with Vectastain Elite kit (Vector Labs.). For detection of TT-reactive T cells, TT was added at 10 μg/ml directly to the PBMCs (1 or 5×10^5 PBMCs/flat-bottomed 96-well plate). Assays were performed on fresh PBMCs. Spots were evaluated and counted using a special computer-assisted video imaging analysis system (Carl Zeiss Vision) as described (16).

Semiquantitative Assessment of CTL Precursors. The multiple microculture method developed by Romero et al. (17) was used to obtain a semiquantitative assessment of CTLp (precursor) specific for Mage-3A1 peptide. Aliquots of frozen PMBCs were thawed and assayed together. CD8$^+$ T cells were isolated with magnetic microbeads (MACS™ separation columns; Miltenyi Biotec) and seeded at 10^4/well in 96-well round-bottomed plates in RPMI 1640 with 10% heat-inactivated human serum. The CD8$^-$ PBMCs were pulsed with peptide Mage-3A1 or the influenza PB1 control peptide VSDGGPNLY (10 μg/ml; 30 min at room temperature), irradiated (30 Gy from a cesium source), and added as an APC population at 10^5/well together with IL-2 (10 IU/ml final) and IL-7 (10 ng/ml final) in a total volume of 200 μl/well. On day 7, 100 μl fresh medium was substituted, and peptide Mage-3A1 or PB1 (1 μg/ml final) and IL-2 (10 U/ml) was added. On day 12, each microwell was divided into three equal samples to test cytolytic activity in a standard 4-h ^{51}Cr-release assay on peptide-pulsed (10 μg/ml for 1 h at 37°C) T2A1 cells, nonpulsed T2A1 cells, and K562 target cells, respectively. All of the assays were performed with an 80-fold excess of nonlabeled K562 to block NK activity. Microwells were scored positive if lysis of T2A1 targets with peptide minus lysis without peptide was ≥12% and this specific lysis was greater than or equal to twice the lysis of T2A1 targets without peptide plus six as described (18). We aimed at testing 30 microwells of 10^4 CD8$^+$ T cells. Therefore, 1/30 positive wells equals at least one CTLp in 3×10^5 (i.e., 30 wells at 10^4 CTLp per well) CD8$^+$ T cells (corresponding to ~3×10^6 PBMCs).

DTH. DTH to Mage-3A1 peptide was assessed by intradermal injection at two sites of each 3×10^5 Mage-3A1 peptide–loaded DC in 0.1 ml PBS. Negative controls were nonpulsed autologous DCs in 0.1 ml PBS and 0.1 ml PBS. DTH to seven common recall antigens (Multitest Merieux) including TT and tuberculin was performed on visits 1, 5, and 8 (Table II).

Assessment and Analysis of Tumor Tissue

For recruitment into the study, Mage-3 gene expression in at least one metastatic deposit had to be demonstrated by RT-PCR as described (14). Accessible superficial skin metastases were removed whenever possible after the vaccinations and subjected to Mage-3 RT-PCR as well as routine histology and immunohistology (to characterize cellular infiltrates).

Statistical Analysis

For analysis of the immune response, pre- and postimmunization values were compared by paired *t* test after logarithmic transformation of the data. Significance was set at $P < 0.05$.

Results

Patient Characteristics

All 13 patients were HLA-A1+, had proven Mage-3 mRNA expression in at least one excised metastasis, and suffered from advanced stage IV melanoma, i.e., distant metastases that were progressive despite chemotherapy and, in some cases, chemoimmunotherapy (Table I). We offered DCs to all patients who fulfilled the inclusion and exclusion criteria, i.e., we did not select for subsets of patients. Two patients (numbers 01 and 03) succumbed to melanoma after two and three vaccinations, respectively. 11 patients received all five planned DC vaccinations in 14-d intervals (Table II) and were thus fully evaluable.

Quality of the Vaccine

All vaccine preparations were highly enriched in mature DCs. More than 95% of the cells were large and veiled in appearance, expressed a characteristic phenotype by flow cytometry (HLA-DR^{+++}CD86^{+++}CD40^{+}CD25^{+}CD14^{-}), and acted as strong stimulators of an MLR at DC/T cell ratios of ≤1:300 (13). Most (mean 80%) expressed the CD83 mature DC marker (19). These features were stable upon removal of cytokines and culture for one to two more days (13). The DCs were pulsed with Mage-3A1 peptide as a tumor antigen and TT or tuberculin as a recall antigen. The latter were internal controls for immunization and possibly provided help for CTL responses (20).

Toxicity

No major (above grade II) toxicity or severe side effects were observed in any patient, including the two patients who were not fully evaluable. We noticed, however, local reactions (erythema, induration, pruritus) at the intracuta-

Table I. *Patients' Characteristics, Status before DC Vaccination, and Response to DC Vaccination*

Patient code	Sex-Age	Onset stage IV	Previous therapy	Metastases at study entry[A]							Clinical Response 14 days after the 5th vaccination	Survival
				regional		distant						
				skin	LN	Skin	LN	Lung	Liver	Other		
Patients with objective tumor regression												
04	M48	1/98	PCI				[illegible]	[illegible]		[illegible]	complete regression of all but 1 lung metastasis, overall progression	10 + >9
06	F61	10/97	CI			[illegible]		[illegible]	[illegible]		complete regression[F] of 1 lung + 4 s.c.[4] metastases, overall progression	8 + >16
07	F48	6/97	C			[illegible]				[illegible]	complete regression[F] of 1 lung[F] + 2 s.c.[4] metastases, overall progression	13 + 12†
08	M67	11/97	PC		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		complete regression[F] of lung + liver + 4 s.c.[4] metastases, overall progression[H]	8 + 3†
09	F43	5/98	C					[illegible]		[illegible]	Partial regression of 1 lung metastasis, overall progression	4 + >11
12	M54	8/98	CI			[illegible]	[illegible]	[illegible]			partial regression of axillary LN metastases, overall progression	28 + >9
Patients without objective tumor regression												
02	F73	5/98	PCI	[illegible]			[illegible]	[illegible]	[illegible]	[illegible]	continuous progression	18 + 5†
05	F49	10/97	CI		[illegible]	[illegible]		[illegible]	[illegible]		continuous progression	5+>17
10	M62	8/98	C	[illegible]		[illegible]					continuous progression	1+ 6†
11	F72	7/98	C		[illegible]	[illegible]		[illegible]		[illegible]	continuous progression	4+ 9†
13	M34	12/97	CI				[illegible]		[illegible]	[illegible]	continuous progression	12 + 5†

Treatment centers: three patients (04, 08, and 12) were treated in Wuerzburg, two in Mainz (patients 10 and 13), and the others in Erlangen.
Pretreatment therapy: PCI, polychemoimmuno. Preceding excisions and radiotherapies are not listed.
Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in millimeters). m, multiple (>3 metastases).
Survival: (since onset of stage IV and as of 5 August 1999) is listed as months since onset stage IV until study entry + number of months since study entry. †Patient deceased.
[4]CNS metastases were regressing at study entry after gamma knife treatment.
[A]Developed (in part) after study entry.
[F]Determined by autopsy.
[H]Sudden death from bleeding into CNS metastasis on visit 8.
[F]The regressions of lung metastases in patients 06 and 07 were documented at a staging 3 mo after visit 8.
mediast., mediastinum; pancr., pancreas.

Table II. *Study Design*

Activities	Screen	Leuka pheresis	Vacc. #1 3 Mio s.c. 3 Mio i.d.	Vacc. #2 3 Mio s.c. 3 Mio i.d.	Vacc. #3 3 Mio s.c. 3 Mio i.d.	Vacc. #4 6 Mio i.v.	Vacc. #5 12 Mio i.v.	Final Evaluation
Clinical visit	1	2	3	4	5	6	7	8
Day	-28/-14	-9	+1	+14	+28	+42	+56	+70
Vaccination			X	X	X	X	X	
Multitest Merieux	X							X
DTH to Mage-3A1 peptide-loaded DC			X		X		X	
Recall-antigen proliferation		X						X
DTLp analysis		X				X		X
ELISPOT Mage-3A1		X	X	X	X	X	X	X
ELISPOT recall antigen		x	x	x	x	x	x	x

X, prespecified in the protocol as obligatory; x, optional.

neous vaccination sites that increased with the number of vaccinations. In 9/11 patients, strong DTH reactions (induration >10 mm in diameter) were noted to DCs carrying a recall antigen (Fig. 1). Elevation of body temperature (grade I and II fever) was observed in most (9/11) patients and was also related to pulsing DCs with recall antigen. The most striking example was patient 02, who progressively developed fever (up to 40°C) upon successive vaccinations but did not show a rise in body temperature when TT was omitted for the final (fifth) vaccination. We observed slight lymph node enlargement, clinically in 63% and by sonography in 83% of patients, after the intracutaneous DC injections. Interestingly, these were delayed, being inapparent during the 2 d of monitoring after vaccinations but detected when patients were investigated again the day before the next vaccination (Table II).

Immunological Responses

Boosting of Recall Antigen-specific Immunity. PBMCs that had been frozen before vaccination and 14 d after vaccination 5 were thawed and assayed together, as specified in the protocol (Table II). In most patients, a significant boost of antigen-specific immunity developed to TT (and tuberculin in patient 10) ($P < 0.004$; Fig. 2). Supernatants from the proliferative assays contained large amounts of IFN-γ (mean 1,679 pg/ml, range 846–4,325) but little IL-4 (IFN-γ/IL-4, 317:1). In five patients, we studied the kinetics of the immune response to TT by IFN-γ ELISPOT analysis. We found an increase after the intracutaneous vaccinations ($P < 0.02$) but a peculiar decrease after the intravenous vaccinations ($P < 0.008$; Fig. 3). Thus, comparing recall immunity before and after all five vaccinations (Fig. 2) as prespecified in the protocol (Table II) obviously underestimated the extent of boosting.

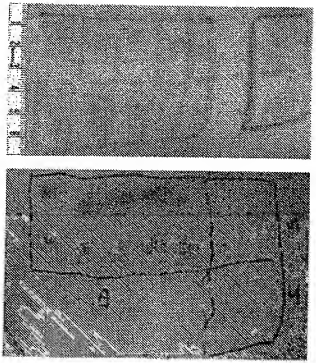

Figure 1. Local reactions to DCs carrying Mage-3A1 peptide and TT at the intradermal and subcutaneous vaccination sites in patient 09 (24 h after vaccination 2; top panel) and 02 (48 h after vaccination 3; bottom panel). Erythema at the i0 intradermal (left) and 2 subcutaneous (right) vaccination sites was followed by induration >10 mm in diameter (with secondary purpura in patient 02). These local reactions represent strong DTH reactions to DCs carrying TT, as such strong reactions did not occur in response to unpulsed DCs or DCs pulsed with Mage-3A1 peptide alone in DTH tests I–III (Table II; reactions not shown).

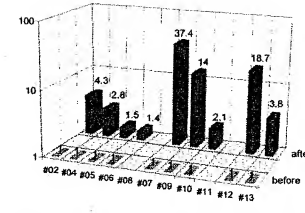

Figure 2. Recall antigen-specific immunity (tuberculin in patient 10; TT in all others) as assayed by antigen-specific proliferation. The cpm values determined after therapy (14 d after vaccination 5) are shown as multiples of pretherapy cpm values. Absolute cpm (cpm with recall antigen minus cpm without antigen) after therapy was 68,917 in patient 02, 85,225 in patient 04, 16,759 in patient 05, 7,913 in patient 06, 16,367 in patient 07, 107,923 in patient 09, 22,790 in patient 10, 4,507 in patient 12, and 1,831 in patient 13 (SEM for all measurements was <20%). Patients 08 and 11 could not be evaluated due to shortage of cells after therapy.

Expansion of Mage-3A1–specific CTLp. Aliquots of PBMCs, frozen before the first and after the third and fifth vaccinations, were thawed at the same time (Table II) and subjected to a semiquantitative recall assay for CTLp (reference 17; Fig. 4). Before vaccination, CTLp frequencies were low or undetectable. In 8/11 patients, we found a significant expansion of Mage-3A1–specific CTLp as indicated by the increase (mean, eightfold; $P < 0.008$) of positive microcultures in the multiple microculture procedure employed for the semiquantitative assessment of CTLp (17). Interestingly, in six patients, the CTLp frequencies were maximal after the three intracutaneous vaccinations ($P < 0.0013$) but then decreased after the two additional intravenous vaccinations in all but one of these patients ($P < 0.026$). Only in 1/11 patients did we observe an increase of CTLp to an irrelevant PB1 influenza peptide that served as a specificity control (not shown).

ELISPOT Analysis for IFN-γ–releasing, Mage-3A1–specific T Cells. We also tried to detect Mage-3A1–specific CTL effectors in uncultured fresh, nonfrozen PBMCs by performing ELISPOT analyses at 14-d intervals on all patients. A significant increase of Mage-3A1–reactive IFN-γ spot–forming cells was apparent only in patients 07 and 09 after the first and second vaccinations, respectively, but the frequency of Mage-3A1–specific effectors was very high (~5,000 and 10,500/10^7 $CD8^+$ T cells; not shown).

DTH Test to Mage-3A1 Peptide–loaded DCs. Tests of DTH to Mage-3A1 peptide–loaded DCs yielded erythema and/or induration (>5 mm diameter) in 7/11 patients (not shown). The results were, however, equivocal due to the frequently observed background to nonpulsed DCs (up to 10 mm in diameter) and the variability from test site to test site.

Clinical Responses

At the end of the trial, i.e., ~2 wk after the fifth vaccination (Table II), we observed temporary growth cessation of some individual metastases, but more intriguingly, in 6/11 patients, complete regression of individual metastases in skin, lymph nodes, lung, and liver (Table I and Fig. 5). Resolution of skin metastases was found in three patients (Table I, patients 06, 07, and 08) and in two of them (06 and 07), it was preceded by local pain, itching, and slight erythema. The six regressing skin lesions of patients 06 and 07 (Table I) were also excised and examined by immunohistology. Clusters of $CD8^+$ T cells were seen around and in the tumor, the latter often necrotic, suggesting an immune attack (Fig. 6).

In patients 06 and 08, the metastases excised at study entry (four and two, respectively) proved to be Mage-3 $mRNA^+$. However, all of the samples removed at the end (two and six, respectively) were Mage-3 $mRNA^-$, suggesting immunoselection for antigen-negative tumor cells. Remarkably, significant infiltration of $CD8^+$ T cells was not found in any of these lesions.

Discussion

In deciding on the source of DCs for this phase I trial, we selected *mature*, monocyte-derived DCs for the follow-

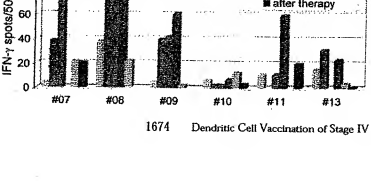

Figure 3. Kinetic analysis of immunity to recall antigens as assessed by TT-specific IFN-γ ELISPOT (SEM for all measurements was <20%). Blood was drawn (see Table II, Study Design) before the first DC vaccination and then every 14 d just before administration of the next DC vaccination (e.g., pre Vacc # 2 means immediately before vaccination 2, i.e., 14 d after vaccination 1), and finally after therapy. Time points at which vaccinations were not performed lack bars. Note the increase after the intracutaneous vaccinations and the decline upon the two vaccinations after intravenous ones. Patient 10, who received tuberculin-pulsed DCs, exhibited no significant change in the TT-specific IFN-γ ELISPOT as expected.

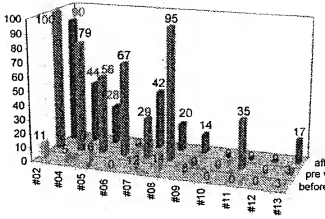

Figure 4. Mage-3A1 CTLp frequency analysis as assessed by semiquantitative recall assay. The y-axis and the numbers above the bars indicate the percentage of positive wells found before vaccination 1, before vaccination 4 (14 d after vaccination 3), and after therapy (usually 14 d after vaccination 5).

ing reasons. Monocyte-derived DCs currently represent the most homogenous and potent DC populations, with several defining criteria and quality controls (12, 13, 21). The method for generating production of these DCs is very reproducible and allows the cryopreservation of large numbers of cells at an identical stage of development (12, 13). Furthermore, these DCs can be produced in the absence of potentially hazardous FCS (12, 13, 21). FCS exposure also leads to large syngeneic T cell responses in culture, so their clinical use (11) might produce nonspecific immunostimulatory effects. Unlike other investigators (9–11), we chose to use mature rather than immature DCs for our first melanoma trial. The DCs that have been used with efficacy in animal experiments were primarily mature (3, 8). Mature DCs are much more potent in inducing CTL and Th1 responses in vitro (reference 22 and Jonuleit, H., A. Gieseke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk, manuscript in preparation), and the DCs are also resistant to the immunosuppressive effects of IL-10 (23) that can be produced by tumors (24–26). Mature DCs also display an extended half-life of antigen-presenting MHC class I (26a) and class II molecules (27). Finally, mature DCs have a high migratory activity (21) and express CCR7 (28), a receptor for chemokines produced constitutively in lymphoid tissues (28). Mature DCs, as used in this cancer therapy trial, have recently also been shown to rapidly generate broad T cell immunity in healthy subjects (28).

Mature DCs were loaded with only one melanoma peptide, Mage-3A1, to avoid uncertainties regarding loading of DCs with multiple peptides (11) of varying affinity and off rate. Successful loading was verified with a Mage-3A1–specific CTL clone and ELISPOT analysis (not shown). The Mage-3A1 peptide (15) was selected for several reasons. It is essentially tumor specific (2) and expressed in tumors other than melanoma (2), and the Mage-3A1 epitope is likely a rejection antigen (14). Moreover, the Mage-3A1 CTLp frequency is exceedingly low in noncancer patients (reference 18; 0.4–3 per 10^7 $CD8^+$ T cells) as well as in cancer patients, even after peptide vaccination (14). Thus, any induction or boost of Mage-3A1 $CD8^+$ T cell responses would indicate a significant superiority in the adjuvant capacities of DCs.

DTH assays with peptide-pulsed DCs were carried out as described by Nestle et al. (11) to detect Mage-3A1 immunity (not shown). However, we did not detect unequivocal DTH. This was due to the frequently observed background to nonpulsed DCs (possibly due to cytokine production by DCs) and the noteworthy variability from test site to test site. As Mage-3A1–specific T cells are $CD8^+$

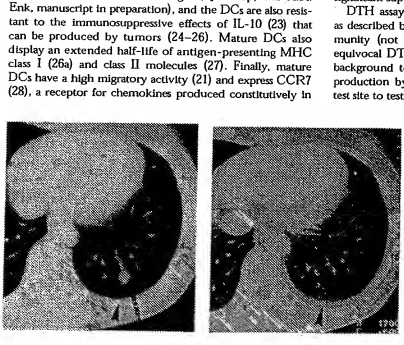

Figure 5. Regression (arrows) of a globular (13 mm in diameter) lung metastasis in patient 07 that was then no longer detectable in serial 6-mm-thick computed tomography scans.

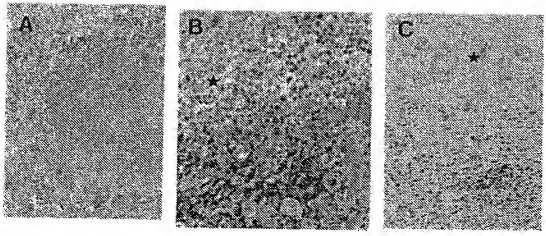

Figure 6. Regressing subcutaneous metastases in patient 06 display a $CD8^+$ lymphocytic infiltrate (alkaline phosphatase/antialkaline phosphatase immunohistochemical staining with anti-CD8 mAb) that surrounds (A) and infiltrates (B) the tumor. Areas of damaged (B, ★) and necrotic (C, ★) melanoma cells are obvious in the vicinity of the $CD8^+$ T cell infiltrate. The metastasis expressed Mage-3, as demonstrated by RT-PCR (data not shown). Magnifications: A, 100; B, 250; C, 160.

T cells and DTH assays typically detect primed $CD4^+$ T cells, we suspect that DTH to MHC class I peptide–pulsed DCs may also for this reason prove not to be a sensitive or reliable way to monitor specific $CD8^+$ T cell–mediated immunity.

In contrast, we found sizable expansions of Mage-3A1–specific CTL precursors in PBMCs from a majority (8/11) of patients ($P < 0.008$; Fig. 4). This is an important proof of the principle of DC-based immunization, and it is also significant from the point of view that tumors can induce tolerance or anergy. It is very promising that CTLp expansions can be induced in far advanced and heavily pretreated stage IV melanoma patients. However, active Mage-3A1–specific effectors were generally not observed in ELISPOT assays, except for in two patients with high frequencies ($>5{,}000/10^7$ $CD8^+$ T cells). Perhaps active $CD8^+$ effectors were rapidly sequestered in the numerous metastases, as suggested by the biopsy studies illustrated in Fig. 6. An alternative explanation is that looking for effectors in peripheral blood 14 d after a preceding vaccination might simply be too late.

Interestingly, in six patients, CTLp had increased to their highest levels after the three intracutaneous vaccinations ($P < 0.0013$) and then decreased ($P < 0.026$) with subsequent intravenous immunizations (Fig. 4). The decrease in CTLp might be due to emigration of activated Mage-3–reactive CTLs into tissues, tolerance induction, or clonal exhaustion via the intravenous route. We also observed decreased responses to recall antigens in the five patients that we studied before and after intravenous vaccination (Fig. 3). The effect of the intravenous route requires additional study, as it may be counterproductive. In contrast, our results clearly demonstrate that the intracutaneous route is effective, so that the less practical intranodal injection propagated by other investigators (11) does not seem essential. It will, however, be necessary to compare subcutaneous and intradermal routes to find out if one is superior.

We found regression of individual metastases in 6/11 patients when patients were staged 14 d after the fifth vaccination (Table I). This percentage of responses was unexpected in far advanced stage IV melanoma patients who were *all* progressive despite standard chemotherapy and even chemoimmunotherapy. In the study by Nestle et al. (11), chemotherapy was only given to 4/16 melanoma patients, and objective tumor responses were observed in 5/16. Therefore, we attribute the regressions to DC-mediated induction of Mage-3A1–specific CTLs. This interpretation is supported by the heavy infiltration with $CD8^+$ T cells of regressing but not nonregressing (skin) metastases. The observation that all of the metastases in patients 06 and 08 that were excised at the end of the study were Mage-3 $mRNA^-$ (whereas those removed at the onset were uniformly positive) suggests immune escape of and selection for Mage-3 antigen–negative tumors. Immune escape might also have been responsible for the lack of tumor response in those nonresponders that had mounted a Mage-3A1–specific CTL response.

After the end of the trial, surviving patients received further vaccinations with DCs and several tumor peptides (Mage-1, tyrosinase, and Mage-3) that were no longer part of the protocol. It is encouraging that 5/11 patients are still alive (Table I) 9–17 mo after study entry, as the expected median survival in patients progressive after chemo(immuno)therapy is only 4 mo (29, 30). One of the initial responders (patient 06) has recently experienced a complete response and has now been disease free for 2 mo. It is interesting that Marchand et al. (14) have also observed that regressions, once they have started, proceed slowly and may take months to complete.

In conclusion, the use of a defined DC vaccine combined with detailed immunomonitoring provides proof that vaccination with mature DCs expands tumor-specific T cells in advanced melanoma patients. In addition, we have found some evidence for the direct interaction between

$CD8^+$ CTLs and tumor cells as well as for escape of antigen-negative metastases. We are convinced that DC-mediated immunization can be intensified further to reveal the presence of expanded populations of effector cells. Efficacy might be increased at the level of the DC, e.g., by optimizing variables such as DC maturational state, route, dose, and schedule or by improving the short life span of DCs in vivo (31, 32); at the level of the T cell, e.g., by providing melanoma-specific $CD4^+$ T cell help (33, 34) or IL-2 (35); and by treating patients earlier in their disease course.

We are grateful to all patients for their confidence and cooperation. We appreciate the support of J. Knop, Head of the Department of Dermatology in Mainz. We thank our colleagues H.B.-R. Balda, H. Hintner, F.S.M. Meurer, and C.R. Neumann for referring patients and T.L. Diepgen for statistical analysis. We are particularly grateful to T. Boon and P. van der Bruggen, who helped us in many ways, and to A. Knuth and T. Woelfel for help in establishing the semiquantitative CTL and ELISPOT assays, respectively. We are obliged to the Protocol Review Committee and Office of Clinical Trials Management of the Ludwig Institute for Cancer Research, in particular H.F. Oettgen and E. Hoffman, for their suggestions on improving the protocol and for supervising the trial.

This work was supported by a grant from the Deutsche Krebshilfe (project #70-2291). B. Thurner was supported by the Training and Mobility of Researchers (TMR) programme of the European Union (EUNIDI).

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Submitted: 1 June 1999 Revised: 21 September 1999 Accepted: 24 September 1999

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APPENDIX 2

Immunosuppressive therapy

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Although Cyclosporin A has improved transplant outcome, its use has serious limitations due to its narrow therapeutic window. New approaches to broaden this window exploit alternative drug formulations, pharmacokinetic profiling and new immunosuppressive agents, such as Rapamycin and Brequinar, which act in a synergistic fashion. There is no evidence to suggest that the pharmacological alternative to Cyclosporin A, FK-506, displays a broader therapeutic window, although it may be tenfold more potent. Similarly, despite the specificity of the IgG2a mouse anti-human CD3 monoclonal antibody, it displays a significant range of clinical side effects, delayed therapeutic action and frequently stimulates generation of human anti-mouse monoclonal antibodies. Recent advances in monoclonal antibody technology seek not only to produce antibodies against determinants involved in alloactivation, but also to 'humanize' the antibodies for reduced side effects. The availability of this array of potential agents highlights the need to develop guidelines for clinical trial methodologies to address the unique needs and demands of organ transplantation.

Current Opinion in Immunology 1992, 4:553–560

Introduction

After thirty years of vigorous but relatively unproductive research, the field of immunosuppressive drugs awakened following the approval of two agents that, in contrast to the non-selective drugs Azathioprine (Aza) and corticosteroids, display relatively specific actions on T cells. One of these, the fungal undecapeptide Cyclosporin A (CsA), not only improved clinical outcomes and broadened the clinical settings in which transplants were successful, but also provided a unique tool for dissecting activation mechanisms leading to lymphokine synthesis. Subsequent approval of the other agent, the IgG2a mouse monoclonal antibody (mAb) OKT-3, heralded the use of reagents that bind selective T-cell surface markers to modulate the immune response. The past decade has witnessed striking progress in the development of new pharmacological agents (Fig. 1). One group inhibits lymphokine biosynthesis, FK 506 or signal transduction, Rapamycin (RAPA). A second group is the nucleotide synthesis inhibitors; Mizorbine [1] and RS61443, a morpholinoethyl-ester analog of mycophenolic acid (MPA) [2••], block purine salvage pathways with the generation of guanosine monophosphate, and the quinoline carboxylic acid Brequinar (BQR) blocks the *de novo* synthesis of pyrimidines [3••]. A third group, new mAbs, recognizes specific surface epitopes on T cells and antigen-presenting cells (Fig. 2). Immunosuppressive activity has been documented [4–9] with several mAbs that bind various determinants as shown in Table 1. Prolonged graft survival has also been achieved with antibodies, or preferably their $F(ab')_2$ fragments, directed towards class I [10] or class II MHC antigens. A refinement of mAb technology is the production of immunotoxins. Ricin α-chain toxin linked to mouse anti-human CD5 IgG1 mAb has been used by Haverty (personal communication) to treat steroid-resistant graft versus host disease in human bone marrow transplantation. This array of new agents proffers an unprecedented opportunity to design effective, yet minimally toxic, regimens to improve the outcome of transplantation in man.

Limitations of existing immunosuppressive regimens

Currently, clinical regimens are based upon the use of CsA, the immunosuppressant benefits of which are seriously limited by side effects. In attempts to augment its efficacy, the corticosteroid Prednisone (Pred), Aza,

Abbreviations

ALG—anti-lymphocyte sera; **Aza**—Azathioprine; **BQR**—Brequinar; **CMV**—cytomegalovirus; **CsA**—Cyclosporin A; **CTL**—cytotoxic T lymphocyte; **DTH**—delayed type hypersensitivity; **ICAM**– intercellular adhesion molecule; **IL** –interleukin; **LFA**—lymphocyte function-associated antigen; **mAb**—monoclonal antibody; **MHC**—major histocompatibility complex; **MPA**—mycophenolic acid; **MZB** – mizorbine; **NF-AT**—nuclear factor of activated T cells; **Pred**—Prednisone; **RAPA**—Rapamycin; **TCR** –T-cell receptor; **Th**—T-helper.

 ISSN 0952-7915

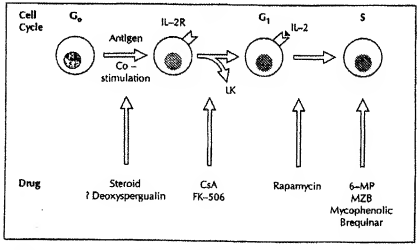

Fig. 1. Classification of immunosuppressive drugs based upon their site of action in the cell cycle. In the first group, corticosteroid, and possibly deoxyspergualin, inhibit antigen- presenting cells. In the second group, Cyclosporin A (CsA) and FK-506 inhibit lymphokine (LK) biosynthesis during the G_0 phase and Rapamycin inhibits signal transduction during the G_1 phase. In the third group (the nucleoside synthesis inhibitors), Mizorbine (MZB) and RS61443 (a morpholinoethyl-ether analog of mycophenolic acid, MPA) inhibit purine synthesis pathways leading to the generation of guanosine monophosphate, whereas Brequinar (quinoline carboxylic acid) inhibits the de novo synthesis of pyrimidines. IL, interleukin; R, receptor.

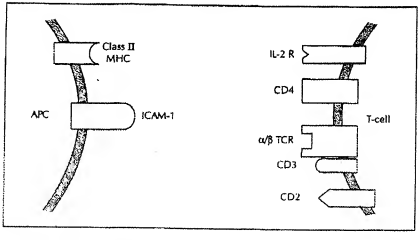

Fig. 2. Epitope targets of monoclonal antibodies. ICAM, intercellular adhesion molecule; IL, interleukin; MHC, major histocompatibility complex; R, receptor; TCR, T-cell receptor.

Table 1. Determinants recognized by immunosuppressive monoclonal antibodies

Monoclonal antibody	Determinant	Reference
IgM T1089.1.A-31	Determinants common to α/β chains of all human T-cell receptors	[4]
IgG2b BMA 031		[5]
OKT4A	CD4	[6]
SDZ CHH 380	CD7	[7]
3B3.1	α chain or α/β complex of interleukin-2 receptor	[8]
BIRR-1	Intercellular adhesion molecule-1 (or CD54)	[9]
2S.3	Lymphocyte function-associated antigen-1	

equine or rabbit polyclonal anti-lymphocyte sera (ALG) and/or mouse OKT-3 mAbs have been combined in empirical regimens that are often tailored to individual patients. To address one limitation of CsA, nephro- and/or hepato-toxicity during the initial post-transplant phase of induction immunosuppressive therapy, which delays allograft and patient recovery, pharmacokinetic control programs have been used either to pre-select CsA doses [11] or to combine ALG or OKT-3 with Aza/Pred in order to delay treatment with CsA. However, immediate, rather than delayed, administration of CsA to patients displaying good initial renal function avoids the disadvantages of ALG/OKT-3 induction, namely an increased risk of cytomegalovirus (CMV) infection, additional expense and/or delayed hospital discharge awaiting satisfactory CsA levels after treatment with CsA. However, patients at extraordinarily high immunological risk, due to rejection of previous allografts within three months, or with marginally-functioning organs may preferably be treated with ALG or OKT-3 induction as a possible means to delay the onset of their first rejection episodes. Two possible alternatives for induction therapy are the CsA analogs that may display reduced nephrotoxicity, namely Cyclosporin G [12], which substitutes norvaline at position 2, and IMM-125 with a hydrophilic substituent at position 9.

To date, no large randomized study has shown that the induction regimen alters the clinical outcome; rather, a small cohort reported by Belitsky *et al.* [13] showed no difference between ALG versus initial CsA therapy, the two options for induction therapy. There may be several reasons for this. Firstly, the polyclonal reagents, such as

rabbit or equine Minnesota ALG and anti-thymocyte globulin, regardless of their source, opsonize T cells, leading to their removal from the circulation. This depletion obviates T-cell mediated attack on the allograft. Among other factors that must be considered is that the central intravenous lines required for polyclonal administration may be accidentally contaminated, producing septicemia. Further, there are no indices of the efficacy of the polyclonal sera. Peripheral blood T-cell numbers above the target range of 50–150/ml tend only to be useful reflections of the appearance of human host anti-equine antibodies, not immunological resistance to therapy. The other induction therapy using OKT-3, which covers and/or modulates CD3 epitopes on the T-cell surface, offers the advantages of both peripheral intravenous administration and readily available fluorescence-activated cell sorting (FACS) tests for antibody efficacy. The clinician monitors patient peripheral blood lymphocyte T-cell epitopes for (a) cells with exposed CD3 epitopes that were not bound by OKT-3 *in vivo* by their capacity to bind fluorescenated OKT-3 *in vitro*, (b) the total number of circulating T cells with fluorescenated anti-CD2, a pan T-cell marker, and (c) the proportion of OKT-3-coated cells detected with a goat anti-mouse IgG reagent. A satisfactory therapeutic effect is observed when the patient has $<25\%$ OKT-3$^+$ cells *in vitro* and 60–75% CD2$^+$T cells, about 40% of which are coated with mouse IgG which binds to OKT-3. While OKT-3 represents an advance in immunosuppressive therapy, it has several serious limitations: (a) severe first-dose reactions, including chills, fever, myalgias and, in the worst cases, pulmonary oedema apparently due to lymphokine release (particularly tumor necrosis factor and interleukin (IL)-2; (b) longer-term adverse effects such as aseptic meningitis; (c) a delay in the therapeutic effect for as long as 7 days after initiation of treatment; (d) induction of human anti-mouse antibodies, generally of the anti-idiotype variety, but not uncommonly of broader reactivity; (e) a frequent incidence of rebound re-rejection episodes upon completion of the therapeutic course; and (f) a tendency toward CMV infections in 40% of treated patients. In addition, both polyclonal and mAb reagents may produce excessive immunosuppression, resulting in increased incidences of CMV infection and/or of lymphomas and other neoplasms, as well as allograft thrombosis. Thus, selection of a CsA versus an antibody induction regimen must balance the risks of nephrotoxicity versus that of excessive immunosuppression.

The use of CsA has reduced the risk of acute rejection, but a rational approach to CsA administration is confused by the tremendous variability between individuals in drug pharmacokinetics and pharmacodynamics [14], which, in turn, generates a fear of irreversible renal injury in case the CsA dose is excessive. Three approaches have been used to address this problem: (a) combining reduced CsA doses with subtherapeutic amounts of Aza [15]; (b) monitoring the parent compound CsA based upon its trough concentration prior to the next drug dose [16]; and (c) adjusting CsA doses prospectively based upon average concentrations calculated from serial measurements of the area under the concentration–time curve [11]. Since drug absorption presents the greatest variability in pharmacokinetics, attempts have been made to increase CsA bioavailability by co-administration of Vitamin E [17]. In addition, the manufacturer has produced a new micro-emulsion formulation that increases the bioavailability by twofold above that of the existing oral solution or capsule preparation, which show equivalent bioavailability in studies comparing both formulations [18]. The critical issue seems to be the drug concentration in the allograft. While direct intra-arterial infusion has been used for experimental models of renal or cardiac allografts, good drug uptake in man can be achieved by presenting CsA as an aerosol in absolute ethanol when it has a mean particle diameter of 1.2 microns [19].

Optimal use of CsA demands the measurement of drug concentrations/activities at the level of its lymphocyte receptor or target signal transduction molecule(s), which may be calcineurin (an enzyme that may be involved in a common step associated with T-cell and IgE receptor signaling pathways) [20••] or the nuclear factor of activated T-cells (NF-AT). However, a major limitation may be the failure of CsA to inhibit lymphocyte activation via the CD28 surface marker [21], an important co-stimulatory pathway that together with T-cell receptor (TCR) stimulation blocks induction of anergy in T-cell clones [22]. Fortunately, rejection episodes under CsA prophylactic therapy tend to be readily reversed by corticosteroid therapy, and the majority of steroid-resistant episodes are overcome with polyclonal ALG and/or OKT-3 therapy. Corticosteroids are believed to represent the Achille's heel of transplantation because of the wide distribution and pleiotropic effects of the glucocorticoid receptor superfamily found in the cytoplasm. These are DNA-binding dimeric transcription factors with a zinc finger structure that recognize enhancer (or negative regulator) elements bearing the GRE motif (GTACAnnnTGTTCT, where n = any nucleotide). One important negative regulatory element is the AP-1 binding site, normally the focus for fos–jun heterodimers [23]. An alternative approach to the reduction of IL-1β generation, an action typical of corticosteroids, is to inhibit the enzyme that cleaves the inactive 31 kD precursor between Asp^{116} and Ala^{117} to release the 153 carboxyl-terminal amino acids that constitute IL-1β [24]. Another immunosuppressive effect may be achieved by the upregulation of the synthesis of transforming growth factor-β by steroids [25]. Withdrawal of steroid treatment months to years after the transplant may be successful in patients who did not reject the transplant [26•].

Preliminary data suggest that a ten-day course of the IgM mouse anti-human α/β TCR mAb T10B9.1A-31 [4], but not BMA031 (C Groth, personal communication), not only produces equivalent therapeutic effects to those of OKT-3, but is less toxic in terms of incidence of fever and neurological and respiratory symptoms, as well as of subsequent infections. Furthermore, T10B9 therapy is not associated with as great a rise in serum creatinine during treatment as OKT-3, suggesting a more rapid attenuation of the allo-immune response. However, the repeated use of xenogeneic antibodies during the induc-

tion phase as well as for anti-rejection therapy may be complicated by the development of neutralizing human anti-mouse antibodies.

A major goal of maintenance immunosuppressive therapy is prophylaxis against chronic rejection. To date, not only has CsA/Pred therapy failed to reduce the incidence of this complication from the 8–10% level observed under the Aza/Pred combination, but there is no way to determine if the failure is due to its inherently modest inhibition of B-cell responses or to physicians' tendency to limit CsA therapy to minimal, possibly ineffective, doses in order to mitigate a renal injury. Thus, despite the improvement in initial graft survival, transplants continue to be lost in the longer term, with half-lives of about seven years for cardiac and 11.5 years for renal transplant in humans. A recent study of the effects of immunosuppressive drugs on coronary vascular disease in heterotopic rat cardiac allografts suggests that RAPA particularly, CsA to a lesser extent, but definitely not FK-506, inhibit pathological endothelial and smooth muscle lesions in arteries and arterioles, which seem to be the critical lesions in the progression of chronic rejection [27].

New pharmacological agents

Both the macrolide FK-506 and the undecapeptide CsA interrupt lymphokine synthesis by inhibiting generation of the Ca^{2+} dependent regulatory proteins NF-AT, NFIL-2A, NFIL-2B, and NF-κβ, but not c-fos, which is necessary for IL-2 generation. Presumably, both drugs also affect serine protease gene transcripts, an excellent marker of rejection [28]. The inhibition of cytotoxic T lymphocytes (CTLs), even in the presence of optimal amounts of IL-2, is a prominent effect of CsA [29] and, apparently, FK-506. Despite the assumption that CsA and FK-506 produce similar inhibitory effects, at least three differences have been observed: first, FK-506 displays a flatter inhibition curve than CsA with a wider discrepancy in potency at the 50% inhibition than at the 95% inhibition level; secondly, CsA leads to the generation of suppressive T cells, whereas FK-506 does not; and thirdly, although both drugs inhibit $CD4^+$ T helper (Th) lymphocytes, which secrete IL-2, only CsA (and not FK-506) permits priming of $CD8^+$CTLs [30]. Furthermore, Bretscher and Havele [31] suggest that CsA switches the immune response to the graft from a delayed type hypersensitivity (DTH) response to an IgG response by inhibiting the Th1 subset with the emergence of the Th2 subset, which actively induces IgG via IL-4 generation and inhibits Th1 cells and DTH via IL-10. Both CsA and FK-506 spare transcription of the down-regulatory lymphokine IL-10. While CsA inhibits transcription of IL-6, this factor is not affected by FK-506.

The coming year should witness publication of a vast array of randomized trials comparing the clinical outcome of liver and renal transplants in patients treated with FK-506 versus CsA. So far, a preliminary non-randomized study of liver recipients showed that FK-506 therapy displays greater neurotoxicity, equivalent nephrotoxicity, but, possibly, less hypertension than does CsA therapy [32••]. A further claim that corticosteroids do not have to be used with FK-506 cannot be assessed due to two factors: firstly, the protocol stipulated higher Pred doses in the CsA cohort than those used with FK-506; and secondly, to date, there is no pharmacokinetic analysis of Pred concentrations in CsA versus FK-506 treatment groups in order to exclude a drug interaction. Additionally, the extremely poor results in the initial study, wherein allegedly CsA-resistant patients were converted to treatment with FK-506, actually reflected antagonism between the two drugs caused by (a) an adverse immunological interaction between the two agents that apparently have similar mechanisms of action [33] and (b) competitive pharmacokinetic interactions. Although FK-506 has not yet been shown to achieve clinical results even equivalent to those of CsA, eventual definition of its relative therapeutic window will depend upon Phase II studies to select well-tolerated drug doses for randomized trials versus CsA therapy.

When a second agent, RS61443, was added in doses of 2 500–3 500 mg per day to a CsA/Pred regimen, it seemed to reduce the incidence of acute rejection episodes. However, these high doses are likely to produce toxicity, particularly leukopenia and gastrointestinal complaints [2••]. Randomized placebo-controlled trials are underway to assess the efficacy of RS61443 versus Aza added to a CsA/Pred regimen. Other studies are examining the impact of a fourth agent, deoxyspergualin, to potentiate an ALG/Aza/Pred/CsA induction protocol.

The studies that claimed Aza displays pharmacological synergism with CsA failed to utilize rigorous experimental design or data analysis [34]. For instance, both *in vitro* analyses [35] and clinical results demonstrate that Aza acts in an additive manner rather than synergistically with CsA [36]. Similarly, *in vitro* analyses suggest that RS61443 [37], mizoribine [37], and thalidomide [38] also act in an additive manner with CsA. Although initial data suggested that BQR potentiates the effect of CsA [3••], recent experiments document true synergism [37]. However, CsA/RAPA combinations show the most impressive degree of synergy both *in vitro* and *in vivo* [39]. Once Phase I toxicity trials have been completed, it will be possible to assess whether BQR or RAPA displays the synergistic effects with CsA in human transplantation that are evident in rodents and large animal models.

New monoclonal antibody reagents

Second generation mAbs are being designed to avoid the severe systemic reactions due to lymphokine release that follow initial doses of OKT3. For example, the IgG2b anti-human α/β TCR mAb BMA 031 used for induction therapy (three 50 mg doses administered on alternate days) delays the onset of first rejection episodes and probably improves one-year graft survival (R Knight and BD Kahan, unpublished data). Similar benefits have been reported with mouse and rat mAbs produced against the activation-induced α-chain, or to new epitopes resulting

from the formation of the αβ complex, of the IL-2 receptor [8].

However, treatment with these antibodies leads to a high incidence of human anti-mouse antibodies, which may attenuate the immunosuppressive effects. Recent work has explored approaches to construct either (a) 'chimeric' antibodies bearing human Fc segments joined to mouse $F(ab')_2$ fragments, or (b) 'humanized' mAbs with mouse idiotypes inserted onto human IgG isotypes (Fig.3). Chimeric antibodies combine the variable regions of mouse antibodies with human antibody constant regions and, therefore, present fewer foreign amino acid sequences to the host. However, one-third of the structure is still of mouse origin. Furthermore, a clinical trial using a chimeric anti-CD7 mAb not only failed to achieve a superior level of immunosuppression induction, but also increased the incidence of vascular thromboses [7]. The latter effect may have been related to the adhesion of Fc receptors on platelets and polymorphonuclear leukocytes to the human Fc regions, bound to endothelium via mouse epitopes. On the other hand, 'humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen combining site, with human variable region frameworks and constant regions. Due to the reduced affinity of 'humanized' antibodies for antigen epitopes, Co *et al* [40•] recommended two innovations: firstly, selection of a human framework that is as homologous to the original mouse antibody as possible; and secondly, insertion of key residues from the mouse model into the construct in order to achieve a molecular conformation that is similar to the native idiotype. The beneficial effects of chimeric and 'humanized' variants of mouse mAbs will be clarified only by randomized clinical trials.

Two alternative approaches seek to utilize mAbs directed against donor MHC antigens or against co-receptor molecules. In a study of non-human primates, OKT-4A IgG2A mAbs, which react with the CD4 co-receptor on Th cells, provoked fewer side effects than OKT-3 [6]. In an initial clinical trial of OKT-4A induction therapy (0.2 mg/kg/day), all six patients suffered rejections. These rejections were reversible, but left residual areas of dead tissue resulting from an obstruction of the blood supply in half the renal allografts. Unfortunately, OKT-4A also generated a strong human anti-mouse antibody response (J Barry, personal communication). Experimental animal models are currently being used to determine if antibody efficacy is related to T-cell depletion and is potentiated by simultaneous treatment with an anti-CD8 mAb. On the one hand, Fathman and colleagues [41] found that depleting anti-CD4 mAbs produced prolonged allounresponsiveness toward allogeneic pancreatic islets, an effect that was moderated by simultaneous treatment with anti $CD8^+$ mAbs, suggesting the role of a regulator $CD8^+$ cell. On the other hand, Waldmann and colleagues [42] induced tolerance toward mouse heart transplants where the donor and recipient were not matched at MHC level using an anti-CD4 mAb that not only did not deplete T cells but also was potentiated by simultaneous administration of an anti-CD8 mAb.

A second approach to co-receptor molecules is based on the interaction of lymphocyte function-associated antigen (LFA)-1 on CTLs with the intercellular adhesion molecule (ICAM)-1 or monocytes. Expression of ICAM-1 is up-regulated following lymphokine release, which occurs during acute allograft rejection but not during other pathological events in the kidney [43••]. Prophylactic and therapeutic administrations of a mAb directed against the high molecular weight α-chain of human ICAM-1 alone delayed both the onset and progression of rejection episodes in primate renal allograft models. Using mouse mAbs directed against LFA-1, Stoppa *et al* [44] reversed steroid resistant acute graft versus host reactions in man. Indeed, the combination of anti-ICAM-1 and anti-LFA-1 mAbs produced allo-tolerance in mice that were not com-

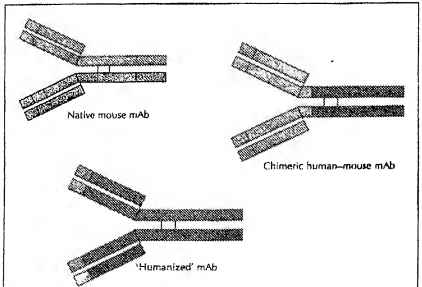

Fig. 3. Types of monoclonal antibody. Chimeric antibodies combine the variable regions of mouse antibodies with human constant regions and, therefore, present fewer foreign amino acid sequences to the host. 'Humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen binding site, with human variable region frameworks and constant regions.

patible at the MHC level [45••]. These promising results in animal models using mAbs directed against T-cell and monocyte co-receptors await confirmation in controlled clinical trials.

While clinical interventions to date have focused on using mAbs directed towards surface epitopes important for the afferent limb of the allo-immune response, there is increasing evidence that anti-idiotypic antibodies, either exogenously introduced or endogenously, spontaneously generated, may regulate the induction of allo-immune responses. A recent study performed by Snider [46] suggested that immunization of hosts with antigen–antibody complexes confers a bias in the epitope, resulting in a less efficient antibody response that shows anti-idiotypic properties. This approach represents a particularly fertile ground for clinical exploration.

Cytokine receptor analogs and antagonists

A new group of immunosuppressive agents are the cytokine receptor antagonists. The discovery and initial testing of an IL-1 receptor antagonist has been reviewed by Arend [47]. IgG-stimulated human monocytes naturally produce IL-1 receptor antagonist, a heterogenous array of glycoproteins of 15–25 kD, depending upon their degree of glycosylation. IL-1 receptor antagonist binds type I, but not type II, IL-1 receptors without activating cells and with considerably less avidity than native IL-1α and IL-1. Type I IL-1 receptors are present on Th2 cells and fibroblasts; Type II IL-1 receptors are present on B cells, neutrophils, and macrophages. Although therapeutic trials of IL-1 receptor antagonist in rheumatoid arthritis and septic shock suggest some beneficial effects, Faherty *et al.* [48] failed to observe that IL-1 receptor antagonist inhibited induction of CTLs, cutaneous DTH, or T-cell dependent humoral antibody responses. They also found that administration of a mAb to type II IL-1 receptor (35F5) was ineffective. Fanslow *et al.* [49•] recently extended their previous studies, which used constructs of the extra-membranous portion of the IL-1 receptor, by using similar constructs of the IL-4 receptor. In the initial studies, they prolonged heterotopic pinnal, neonatal mouse heart allograft survival, but failed to prevent allo-sensitization, as documented by a rapid, secondary-type proliferative response upon *in vitro* one-way mixed lymphocyte reactions. In their recent studies, constructs of IL-4 receptor alone, or in combination with rat anti-mouse IL-4 receptor mAb, induced modest prolongation of heart allo-explants.

Immunosuppressive drug trials

Of the numerous obstacles currently hindering the development of efficacious immunosuppressive regimens, the lack of methodology for clinical transplantation trials is of particular importance. To date, no series of Phase I and II toxicity and dose-finding trials has been conducted in order to establish a foundation for clinical investigation. The introduction of Aza, steroids, and CsA, as well as the preliminary trials of FK-506, have relied upon empirical approaches. Important obstacles to comprehensive trials include the relatively small numbers of transplant cases, the use of unrefined end-points such as graft and patient survival, and the lack of well-established criteria for the diagnosis and grading of rejection episodes, deficits that obfuscate the use of this event as an intermediate endpoint. In addition, no *in vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficacy; hence, there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from *in vitro* systems to *in vivo* conditions.

Since present results with CsA-based regimens yield excellent graft survivals, extremely large numbers of patients must be entered into clinical trials to document improved efficacy of a new agent. Even more extensive efforts will be needed to exclude the possibility that the results with the new agent are not actually worse than those obtained with the existent CsA regimen. In light of the presently high success rates, the benefits of any new regimen must be based upon both the potency and the mitigation of side effects, as assessed by quantitative parameters, including glomerular filtration rates. The practice of clinical research in transplantation must proceed to develop principles of rigorous study design and precise analytical tools in order to most expeditiously evaluate the available array of new immunosuppressants described in this review.

Acknowledgements

This study was supported by an award from the National Institute of Diabetes, Digestive and Kidney Diseases NIDDK38016-05. The author acknowledges the invaluable editorial assistance of Ms. B. Lee Ligon.

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APPENDIX 3

0041-1337/99/6711-1453/0
TRANSPLANTATION

Vol. 67, 1453–1460, No. 11, June 15, 1999
Printed in U.S.A.

INTERLEUKIN-12 (IL-12)-DRIVEN ALLOIMMUNE RESPONSES IN VITRO AND IN VIVO

REQUIREMENT FOR β1 SUBUNIT OF THE IL-12 RECEPTOR[1]

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Background. **Interleukin-12 (IL-12) mediates its biologic activities via binding high-affinity receptors on T and natural killer cells. Although emphasis has been placed on the requirement for IL-12Rβ2 in IL-12 bioactivity, the role of IL-12Rβ1 is less well defined. The current study evaluated the effects of exogenous IL-12 on alloantigen-specific immune responses and determined the requirement for IL-12Rβ1 in IL-12-mediated alloimmunity.**

Methods. **The mouse heterotopic cardiac transplant model was employed to evaluate the effects of IL-12 on alloantigen-specific immune responses in vivo. In addition, IFN-γ production in mixed lymphocyte cultures (MLC) supplemented with IL-12 was measured to assess the effects of IL-12 on Th1 function in vitro. Mice deficient in IL-12Rβ1 (IL-12Rβ1$^{-/-}$) were used to determine the requirement for this receptor component in IL-12-driven alloimmune responses.**

Results. **Addition of IL-12 to MLC consisting of wild-type splenocytes enhanced alloantigen-specific proliferative responses and Th1 development. In contrast, IL-12 did not alter these in vitro immune parameters in IL-12Rβ1$^{-/-}$ MLC. Treatment of wild-type cardiac allograft recipients with IL-12 resulted in high concentrations of serum interferon-γ (IFN-γ) and a 10-fold increase in IFN-γ production by recipient splenocytes after restimulation in vitro. However, this fulminate Th1 response did not accelerate allograft rejection. Importantly, IL-12 had no effect on serum IFN-γ or in vivo priming of Th1 in IL-12Rβ1$^{-/-}$ recipients. Finally, administration of IL-12 to WT allograft recipients resulted in a bimodal alloantibody response: antibody production was suppressed at high doses of IL-12, and enhanced at lower doses.**

Conclusions. **IL-12 markedly enhances alloantigen-specific immune function; however, these exaggerated Th1-driven responses do not culminate in accelerated allograft rejection. Further, these data indicate that IL-12Rβ1 is essential for the enhancement of both in vitro and in vivo alloimmune responses by exogenous IL-12.**

It is well established that interleukin-12 (IL-12*) is a critical cytokine involved in the regulation of Th1- and Th2-mediated immune responses in several experimental models (reviewed in *1* and *2*). IL-12 has direct stimulatory and inhibitory effects on Th1 and Th2, respectively (*3–6*). Further, this cytokine promotes Th1 and inhibits Th2 development indirectly by inducing interferon-γ (IFN-γ) production by activated T cells and natural killer cells (*7–12*). Th1 have been accepted as key regulators of allograft rejection, in that this cell type promotes both delayed-type hypersensitivity and cytotoxic T lymphocyte responses, which are believed to be the principle terminal effector mechanisms of acute allograft rejection (*13*, *14*). An understanding of the role of IL-12 in graft rejection is just emerging. For example, IL-12 clearly augments alloreactive Th1 development in vitro (*15*). However, the presence of IL-12 is not mandatory for the development of acute cardiac allograft rejection (*15*, *16*). Hence, an important question is whether enhanced Th1 function alters the rejection response. Given the IL-12/Th1 dogma, one would predict that IL-12 would augment alloreactive Th1 function, resulting in accelerated allograft rejection. The present study therefore was designed to test the hypothesis that IL-12-driven Th1 responses would exacerbate cardiac allograft rejection.

IL-12 mediates its biologic effects by interacting with a high-affinity receptor, which consists of at least two cloned components, IL-12Rβ1 and IL-12Rβ2 (*17–19*). IL-12Rβ1 interacts with the p40 subunit of IL-12, whereas the p35 subunit of IL-12 is believed to bind to IL-12Rβ2 (*19*, *20*). Emphasis has been placed on the necessity for IL-12Rβ2 in IL-12 signaling (*21*, *22*). However, by utilizing IL-12Rβ1 knockout mice (IL-12Rβ1$^{-/-}$), Wu et al. (*23*) recently reported that the β1 subunit of IL-12R is essential for IL-12-driven proliferation and IFN-γ production by mitogen-activated blasts, natural killer cell lytic activity, and IFN-γ production in response to endotoxin. We have reported that the p40 subunit of IL-12 stimulates alloreactive CD8+ Th1 development both in vitro (*24*) and in vivo (*15*). These observations suggest that

[1] This work was supported by grant R01 AI31946 (D.K.B.) from the National Institutes of Health.

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* Abbreviations: Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; GVHD, graft-versus-host disease; H&E, hematoxylin and eosin; IFN-γ, interferon-γ; IL, interleukin IL-12Rβ1$^{-/-}$, mice deficient in β1 subunit of IL-12 receptor; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; WT, wild-type.

IL-12Rβ1 may be needed for alloreactive Th1 development, and that signaling through IL-12Rβ1 may be sufficient to mediate IL-12's biologic activity on CD8+ T cells. Hence, the present study employed IL-12Rβ1$^{-/-}$ mice to determine whether β1 subunit of IL-12R is required for IL-12-induced alloantigen-specific immune responses. To our knowledge, this study is the first to investigate the effects of IL-12 treatment on alloreactive Th1 development in vivo and to establish a mandatory role for IL-12Rβ1 in IL-12-driven alloimmune responses.

MATERIALS AND METHODS

Mice. Wild-type (WT) C57BL/6 and BALB/c mice between 6 and 12 weeks of age were obtained from Charles River Laboratories (Raleigh, NC). Generation of C57BL/6 IL-12Rβ1$^{-/-}$ mice has been described previously (23). These mice were generated on the 129/Sv background and back-crossed to C57BL/6 mice for five generations, then intercrossed to generate homozygotes.

Medium. The culture medium used in these studies was Dulbecco's minimum essential medium supplemented with 1.6 mM L-glutamine, 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μM folic acid, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, 2% fetal calf serum (all obtained from Life Technologies, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical, St Louis, MO).

Mitogen-driven cytokine production. To investigate the requirement for the β1 subunit of IL-12R in mitogen-stimulated IL-10 and IFN-γ production, splenocytes (2×10^6 cells/ml) isolated from naive WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were incubated for 72 hr with 1 μg/ml concanavalin A (Con A) (Sigma Chemical). Cultures were supplemented with 1 ng/ml murine recombinant IL-12 (rIL-12) (kindly provided by Dr. Maurice Gately, Hoffmann-La Roche Inc.) to assess the effect of exogenous IL-12 on Con A-stimulated cytokine production by splenocytes of WT and IL-12Rβ1-deficient mice. Resulting supernatants were harvested at 72 hr, and the concentrations of IL-10 and IFN-γ measured by enzyme-linked immunosorbent assay (ELISA).

In vitro alloimmune responses. To assess alloantigen-specific Th1 development, splenocytes (1×10^6 cells/ml) isolated from naive WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated (5000 rads) BALB/c splenocytes (1×10^6 cells/ml). Where indicated, 1 ng/ml of murine rIL-12 was added to primary mixed lymphocyte cultures (MLC) to assess the effect of exogenous IL-12 on alloantigen-driven Th1 function and to evaluate whether Th1 from IL-12Rβ1-deficient mice were responsive to IL-12 stimulation. The concentration of rIL-12 was selected from dose-response experiments in which the amount of rIL-12 needed for maximal enhancement of alloantigen-specific proliferation was 5–10 ng/ml (data not shown). Resulting cell populations were harvested, washed three times, and restimulated (at 1×10^6 cells/ml) with irradiated BALB/c stimulator cells (1×10^6 cells/ml). MLC supernatants were collected after 24 hr (IL-4 and IL-10) or 72 hr (IFN-γ), and cytokine concentrations measured by ELISA.

In addition, splenocyte proliferative response to alloantigens was determined in cultures either left unmodified or supplemented with 1 ng/ml murine rIL-12. WT or IL-12Rβ1$^{-/-}$ C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated for 5 days with irradiated BALB/c splenocytes (1×10^6 cells/ml) in 96-well U-bottom plates (Becton Dickinson, Lincoln Park, NY) in a final volume of 200 μl (done in quadruplicate). Cultures were pulsed with 0.5 μCi/well [methyl-^{3}H]thymidine (ICN, Costa Mesa, CA) for the final 8 hr of the incubation period. [methyl-^{3}H]Thymidine incorporation was assessed on a Wallac 1205 Betaplate scintillation counter (Wallac, Turku, Finland).

Heterotopic cardiac transplantation. Intact BALB/c ($H2^d$) hearts were anastomosed to the great vessels in the abdomens of WT or IL-12Rβ1$^{-/-}$ C57BL/6 ($H2^b$) mice as described by Corry et al. (25). In this model, the transplanted heart is perfused with the recipient's blood and resumes contractions until acutely rejected, which occurs in unmodified WT recipients of this strain combination in approximately 8–9 days (15, 24). Graft function was evaluated by daily abdominal palpation. Myocyte damage and intensity of graft-infiltrating cells were assessed by routine hematoxylin and eosin (H&E) staining of paraffin-embedded sections of transplanted allografts.

Experimental groups. Cardiac allograft recipients were divided into four groups: (1) recipients injected intraperitoneally with 1 mg of anti-CD8 monoclonal antibody (mAb) (hybridoma 2.43, purified by Montana ImmunoTech Inc., Bozeman, MT) on days −2 and −1 before transplantation, (2) animals given daily intraperitoneal injections of murine rIL-12 (0.1 or 1.0 μg) on days 1–6 after transplantation, (3) recipients injected with a combination of 2.43 anti-CD8 mAb plus rIL-12, and (4) unmodified (no treatment) mice, which served as controls. Depletion of CD8+ cells (<2%) was verified by flow cytometry using anti-CD8:fluorescein isothiocyanate antibody (PharMingen).

In vivo alloimmune responses. To monitor in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from allograft recipients were restimulated with irradiated BALB/c stimulator cells (1×10^6 cells/ml), and the concentration of IFN-γ was measured by ELISA. As an additional measure of the in vivo activity of IL-12 on IFN-γ production, sera IFN-γ concentrations in WT and IL-12Rβ1$^{-/-}$ cardiac allograft recipients were measured by ELISA. Further, to assess the effect of IL-12 treatment on B cell function, sera alloantibody (IgM, IgG1, and IgG2a) levels were determined (see below).

Cytokine ELISA. Experimental samples (100 μl) were added in triplicate to plates coated with 5 μg/ml rat anti-mouse IFN-γ, IL-4, or IL-10 capture antibodies (PharMingen). Standards were employed by preparing 2-fold dilutions of murine recombinant IFN-γ, IL-4, and IL-10 (PharMingen), with a starting concentration of 25, 2.5, and 10 ng/ml, respectively. After a 1-hr incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS. One hundred microliters of rat anti-mouse secondary biotinylated antibodies (1 μg/ml) (PharMingen) was then added, and plates were incubated at room temperature for 45 min. Plates were then washed three times with 0.05% Tween 20 in PBS, and 100 ml of avidin-peroxidase (Sigma Chemicals) was added. After a 30-min incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS, and 100 ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma Chemical) was then added to each well. After 20 min, absorbance was determined at 405 nm by an EL 800 microplate reader (Bio-Tek Instruments, Winooski, VT). Sample cytokine concentrations were calculated from a standard curve. The sensitivity of this assay is approximately 300 pg/ml for IFN-γ, 100 pg/ml for IL-4, and 150 pg/ml for IL-10.

Sera alloantibody determination. P815 cells ($H2^d$) were stained for flow cytometric analysis using dilutions of sera (1:50) obtained from cardiac allograft recipients as the primary antibody, followed by fluorescein isothiocyanate-conjugated isotype-specific anti-mouse IgM, IgG1, and IgG2a secondary antibodies (The Binding Site, San Diego, CA). Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACScan.

Statistics. Statistical analyses in this study were done using a Student's *t* test performed by the program StatView 4.1.

RESULTS

Requirement for IL-12Rβ1 in T Cell Responses in Vitro

Enhancement of mitogen-driven IFN-γ and IL-10 production by IL-12 requires IL-12Rβ1. IL-12 stimulates concomitant production of IL-10 and IFN-γ by activated T cells (15, 26, 27). To determine whether β1 subunit of IL-12R is required for production of these cytokines, C57BL/6 splenocytes isolated WT or IL-12Rβ1-deficient mice were stimu-

lated with Con A for 72 hr, and supernatant cytokine concentrations were determined by ELISA. Production of the Th1 cytokine IFN-γ by Con A-stimulated splenocytes isolated from IL-12Rβ1$^{-/-}$ mice was readily detectable (Fig. 1A), although concentrations were lower than that seen in WT controls (IL-12Rβ1$^{-/-}$ = 1.03 ng/ml vs. WT = 5.24 ng/ml). Addition of exogenous rIL-12 significantly enhanced IFN-γ production by mitogen-stimulated splenocytes obtained from WT mice (15.79 ng/ml). In contrast, IFN-γ production by splenocytes from IL-12Rβ1$^{-/-}$ mice was not altered after the addition of rIL-12 (1.54 ng/ml).

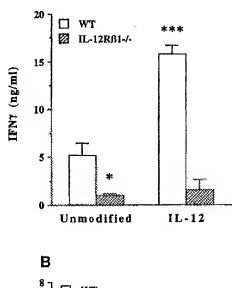

FIGURE 1. Mitogen-driven cytokine production by splenocytes isolated from IL-12Rβ1$^{-/-}$ mice. Splenocytes (2×10^6 cells/ml) obtained from WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were stimulated in vitro with 1 μg/ml Con A. Cultures were either left untreated or supplemented with murine rIL-12 (1 ng/ml). Supernatants were collected after 72 hr, and the concentrations of IFN-γ (A) and IL-10 (B) were determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples ± SD. Data are representative of three separate experiments. In panel A, *, $P<0.05$ (WT unmodified vs. IL-12Rβ1$^{-/-}$ unmodified); ***, $P<0.005$ (WT unmodified vs. WT IL-12-treated). In panel B, *, $P<0.05$ (WT unmodified vs. WT IL-12-treated).

The requirement for IL-12Rβ1 in IL-12-driven IL-10 production also was assessed. Splenocytes isolated from IL-12Rβ1-deficient mice produced similar levels of IL-10 upon Con A stimulation when compared to WT cells (Fig. 1B). rIL-12 enhanced Con A-stimulated IL-10 production by WT splenocytes (2.70 ng/ml vs. 5.65 ng/ml). However, the β1 subunit of IL-12R was required for this response, as IL-12 did not affect IL-10 secretion by mitogen-stimulated IL-12Rβ1$^{-/-}$ splenocytes.

In vitro alloreactive T helper cell development. To evaluate the requirement for IL-12Rβ1 in IL-12-driven alloantigen-specific T cell development, naive splenocytes obtained from WT or IL-12Rβ1$^{-/-}$ mice were incubated for 5 days with irradiated BALB/c splenocytes in primary MLC, which were either left unmodified or supplemented with rIL-12. Resulting cell populations were restimulated with irradiated BALB/c splenocytes in the absence of rIL-12, and in vitro IFN-γ, IL-4, and IL-10 production determined by ELISA (Table 1). Primed WT splenocytes secreted high levels of IFN-γ upon restimulation with alloantigens. Splenocytes obtained from IL-12Rβ1$^{-/-}$ mice secreted IFN-γ upon restimulation with alloantigens, albeit to a lesser degree than WT cells (WT = 21.32 ng/ml vs. IL-12Rβ1$^{-/-}$ = 5.72 ng/ml). The decrease in alloantigen-stimulated IFN-γ production in IL-12Rβ1-deficient mice was not associated with a decrease in the cells' ability to proliferate in response to alloantigens (Fig. 2), in that [methyl-^{3}H]thymidine incorporation by alloantigen-stimulated IL-12Rβ1$^{-/-}$ splenocytes was similar to that seen by WT cells (IL-12Rβ1$^{-/-}$ = 13,385 cpm vs. WT = 11,441 cpm). In both groups, IL-4, IL-10 (Table 1), and IL-5 (data not shown) were not detected in cultures that were not supplemented with exogenous rIL-12.

As shown in Table 1, exogenous rIL-12 markedly enhanced IFN-γ production by WT splenocytes in vitro (21.32 ng/ml vs. 215.13 ng/ml), but failed to augment IFN-γ secretion by cells obtained from IL-12Rβ1$^{-/-}$ mice (5.72 ng/ml vs. 6.80 ng/ml). Likewise, rIL-12 significantly enhanced WT splenocyte proliferation in the MLC (Fig. 2), but did not alter the proliferative ability of splenocytes isolated from IL-12Rβ1-deficient mice. Finally, the addition of exogenous rIL-12 to cultures

TABLE 1. IL-12 does not enhance alloantigen-specific Th1 development in IL-12Rβ1$^{-/-}$ mice in vitro*

Treatment	IFN-γ (ng/ml)	IL-4 (ng/ml)	IL-10 (ng/ml)
Unmodified			
WT	21.32±0.88	ND	ND
IL-12Rβ1$^{-/-}$	5.72±0.24**	ND	ND
IL-12 (1 ng/ml)			
WT	215.13±13.52***	ND	1.54±0.21
IL-12Rβ1$^{-/-}$	6.80±0.21	ND	ND

* Splenocytes (1×10^6 cells/ml) obtained from WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated allogeneic splenocytes (1×10^6 cells/ml) in unmodified MLC or MLC supplemented with murine rIL-12 (1 ng/ml). Resulting cell populations were harvested and restimulated with alloantigens for cytokine determination. Supernatants concentrations of IFN-γ (72 hr), IL-4 (24 hr), and IL-10 (24 hr) were measured by ELISA. Results are expressed as the cytokine concentration in triplicate samples (mean ± SD). Data are representative of four separate experiments. ND indicates not detectable. **, $P<0.01$, WT unmodified vs. IL-12Rβ1$^{-/-}$ unmodified; ***, $P<0.005$, WT unmodified vs. WT IL-12-treated.

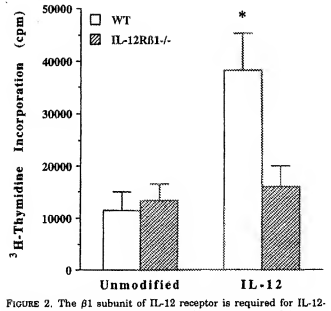

FIGURE 2. The β1 subunit of IL-12 receptor is required for IL-12-induced stimulation of alloantigen-specific splenocyte proliferation. C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated with irradiated allogeneic BALB/c splenocytes (1×10^6 cells/ml) in 96-well microtiter plates for 5 days. Cultures were pulsed with 0.5 μCi/well [methyl-^{3}H]thymidine for the final 8 hr of the incubation period, and thymidine incorporation was determined by liquid scintillation spectrophotometry. Results are expressed as the mean cpm in quadruplicate samples ± SD. Data are representative of three separate experiments. *, $P<0.05$ (WT unmodified vs. WT IL-12-treated).

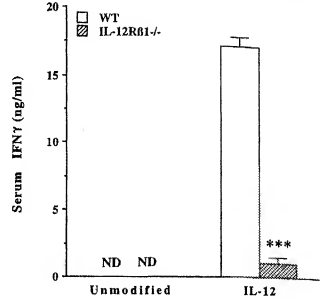

FIGURE 3. Treatment of WT, but not IL-12Rβ1$^{-/-}$ allograft recipients with rIL-12 markedly increases serum IFN-γ. WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice bearing BALB/c cardiac allografts were either left untreated or given daily intraperitoneal injections of 1.0 μg of rIL-12 on days 1–6 after transplantation. On day 7, blood obtained from allograft recipients was pooled and serum collected after centrifugation. Serum IFN-γ was determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples ± SD. Data are representative of three independent experiments. ND indicates not detectable. ***, $P<0.005$ (WT IL-12-treated vs. IL-12Rβ1$^{-/-}$ IL-12-treated).

stimulated the secretion of IL-10 by alloantigen-stimulated WT splenocytes, but not IL-12Rβ1$^{-/-}$ cells (Table 1). Collectively, these data indicate that the β1 subunit of IL-12R is required for the enhancement of several in vitro alloimmune responses by exogenous rIL-12, including increased alloantigen-stimulated T cell proliferation, and IFN-γ and IL-10 production.

Effects of Exogenous IL-12 on Alloimmune Responses in Vivo

Enhancement of serum IFN-γ by IL-12 treatment. To monitor the in situ effects of IL-12 treatment on IFN-γ production in cardiac allograft recipients, serum IFN-γ concentrations were measured on day 7 after transplantation (Fig. 3). In both WT and IL-12Rβ1$^{-/-}$ allograft recipients, serum IFN-γ was undetectable by ELISA on day 7 after transplantation. Treatment of WT recipients with rIL-12 markedly increased serum IFN-γ in three independent experiments; however, this treatment regimen had little effect on the concentration of serum IFN-γ in IL-12Rβ1$^{-/-}$ allograft recipients.

Effects of IL-12 on alloantigen-specific Th1 development in vivo. Splenocytes obtained from cardiac allograft recipients were restimulated in vitro with donor alloantigens and supernatant concentrations of IFN-γ were determined by ELISA. This assay detects in vivo primed Th1, in that splenocytes from naive, nontransplanted mice produce minimal or undetectable levels of IFN-γ under these conditions (*15, 16, 24*). Restimulation of splenocytes from unmodified IL-12Rβ1$^{-/-}$ allograft recipients with donor alloantigens resulted in the secretion of similar amounts of IFN-γ compared to that seen in WT recipients (IL-12Rβ1$^{-/-}$ = 7.16 ng/ml vs. WT = 6.24 ng/ml) (Fig. 4). Treatment of WT recipients with IL-12 resulted in a 10-fold increase in the production of IFN-γ (59.05 ng/ml). In contrast, IFN-γ production by splenocytes obtained from IL-12Rβ1$^{-/-}$ recipients treated with IL-12 in vivo was similar to untreated values (9.59 ng/ml), indicating that the β1 subunit of IL-12R is required for IL-12-mediated enhancement of in vivo sensitization of IFN-γ-producing cells. Further, these results indicate that in vivo Th1 development can occur in a state of IL-12 unresponsiveness.

Effects of exogenous IL-12 on cardiac allograft rejection. As IL-12 treatment markedly enhanced Th1 responses in WT allograft recipients (Figs. 3 and 4), one might predict that IL-12 treatment would exacerbate allograft rejection. To test this possibility, cardiac allograft function was monitored by daily abdominal palpation in WT or IL-12Rβ1$^{-/-}$ allograft recipients bearing BALB/c hearts. Cardiac allograft recipients were either left untreated or injected once daily with 1.0 μg of rIL-12. Treatment of WT allograft recipients with this dose of rIL-12 (n = 10) resulted in symptoms of cachexia including weight loss (mean decrease = 2.0 ± 0.7 g in 1 week), ruffed fur, hunched posture, and decreased activity. In contrast, IL-12Rβ1$^{-/-}$ allograft recipients exhibited no signs of IL-12-induced toxicity.

The mean cardiac allograft survival in unmodified WT recipients was approximately 8 days (data not shown; *15, 24*).

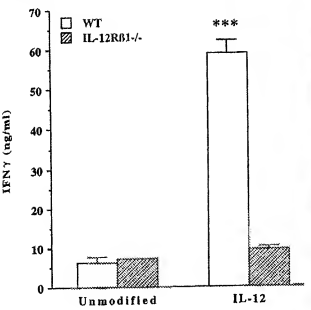

FIGURE 4. Enhancement of in vivo sensitization of IFN-γ-producing cells by IL-12 requires IL-12Rβ1. WT or IL-12Rβ1$^{-/-}$ C57BL/6 cardiac allograft recipients were either left untreated or injected daily with 1.0 μg of rIL-12 on days 1–6 after transplantation. To assess in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from cardiac allograft recipients were restimulated with irradiated BALB/c splenocytes (1×10^6 cells/ml). Supernatants were collected after 72 hr, and the concentration of IFN-γ was determined by ELISA. Results are expressed as the mean concentration of IFN-γ in triplicate samples ± SD. Data are representative of three separate experiments. ***, $P<0.005$ (WT unmodified vs. WT IL-12-treated).

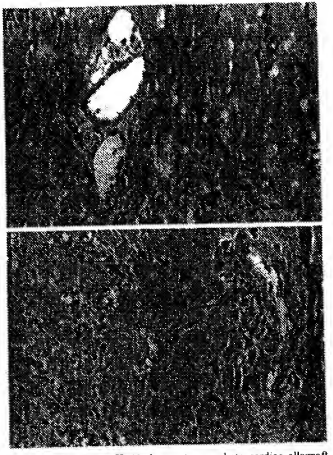

FIGURE 5. Exogenous IL-12 does not exacerbate cardiac allograft rejection. C57BL/6 WT recipients of BALB/c cardiac allografts were either left untreated or injected intraperitoneally with murine rIL-12 (1.0 μg) on days 1–6 after transplantation. On day 7, allografts were harvested for histologic evaluation. (A) H&E-stained section of allografts from WT recipients left untreated (original magnification, ×400). (B) H&E-stained section of allografts from WT recipients treated with rIL-12 (original magnification, ×400). Note in both experimental groups moderate mononuclear cell infiltrates, and relative health of myocytes and vessels. These characteristics are associated with the early phase of acute rejection before onset of myocyte necrosis and vascular damage, which is observed on days 8 or 9 after transplantation. Results are representative of at least 10 individual transplants for each experimental group.

Cardiac allografts in IL-12Rβ1$^{-/-}$ recipients were rejected in a similar fashion to that seen in IL-12-deficient mice (*15*), in that grafts were uniformly rejected by day 7 (n=8). As expected, treatment of IL-12Rβ1$^{-/-}$ allograft recipients with rIL-12 had no effect on the tempo of allograft rejection (n=6). Interestingly, despite the overwhelming Th1 response induced by rIL-12 in WT allograft recipients (Figs. 3 and 4), treatment of these animals with rIL-12 did not appear to accelerate the tempo of graft rejection when compared to grafts of untreated WT recipients on day 7 after transplantation. For example, 7 of 10 (70%) allografts of WT recipients treated with rIL-12 were still functioning on day 7. A histologic evaluation of these grafts revealed similar parameters of early rejection compared to unmodified WT recipients. Specifically, histology was characterized by diffuse mononuclear cell infiltrates, viable myocytes as evidenced by visible nuclei, and relatively uninvolved vessels (Fig. 5). Hence, rIL-12 treatment did not accelerate the pathologic changes associated with acute rejection.

Phenotype of alloantigen-reactive Th1 in WT allograft recipients treated with rIL-12. To determine the phenotype of Th1 responsive to exogenous rIL-12, WT cardiac recipients were depleted in vivo of CD8+ T cells (Fig. 6). Splenocytes obtained from CD8 depleted cardiac allograft recipients produced markedly less IFN-γ upon in vitro restimulation with irradiated donor splenocytes (WT unmodified=6.15 ng/ml vs. WT anti-CD8 mAb-treated=0.52 ng/ml). Similarly, Th1 that develop as a result of IL-12 stimulation in these experiments were predominantly CD8+ T cells (Fig. 6), as depletion of CD8 cells resulted in a reduction in IFN-γ production (WT IL-12-treated=35.89 ng/ml vs. WT IL-12 plus anti-CD8 mAb-treated=2.75 ng/ml).

IL-12 Treatment (1.0 μg/Day) Inhibits Alloantibody Responses

Given our findings that treatment of WT cardiac allograft recipients with rIL-12 resulted in significant augmentation of serum IFN-γ (Fig. 3) and in vivo priming of Th1 (Fig. 4), one would predict that IL-12 treatment should drive IgG2a alloantibody production. To test this possibility, sera alloantibody production was assessed on day 7 after transplanta-

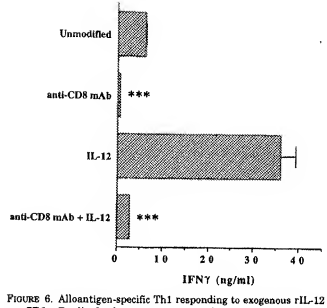

FIGURE 6. Alloantigen-specific Th1 responding to exogenous rIL-12 are CD8+ T cells. In these experiments, splenocytes were obtained from cardiac allograft recipients either left untreated or treated with anti-CD8 mAb, rIL-12, or a combination of anti-CD8 mAb plus rIL-12. Th1 function was assessed by IFN-γ production after a 72-hr restimulation of recipient's splenocytes with irradiated BALB/c splenocytes. Results are expressed as the mean concentration of IFN-γ in triplicate samples ± SD. Data are representative of three separate experiments. ***, $P<0.005$ (WT unmodified vs. WT anti-CD8 mAb-treated; WT IL-12-treated vs. WT IL-12 plus anti-CD8 mAb-treated).

tion in WT cardiac allograft recipients either left unmodified or treated once daily with 1.0 μg of rIL-12. In these experiments, sera IgG2a was undetectable in rIL-12-treated WT allograft recipients at this time point (data not shown). Further, treatment of WT recipients with rIL-12 resulted in reduced sera IgM alloantibody in three independent experiments, compared to untreated WT recipients (Table 2). This observation indicates that high doses of rIL-12 inhibit, rather than enhance, alloantibody production in this model. In contrast, treatment of IL-12Rβ1$^{-/-}$ allograft recipients with rIL-12 resulted in a slight increase in sera IgM compared to unmodified knockout recipients.

TABLE 2. High-dose rIL-12 inhibits IgM production in vivo[a]

	Serum IgM (mean channel fluorescence)	
	Unmodified	IL-12 Treatment
WT		
Experiment 1	37.18	9.85
Experiment 2	33.97	11.39
Experiment 3	78.51	27.37
IL-12Rβ1$^{-/-}$		
Experiment 1	17.36	31.52
Experiment 2	33.83	43.64
Experiment 3	21.67	35.96

[a] Serum was obtained on day 7 after transplantation from WT or IL-12Rβ1$^{-/-}$ cardiac allograft recipients. Animals were either left untreated or injected with 1 μg of rIL-12 once daily. Anti-BALB/c IgM was assessed by flow cytometry using P815 ($H2^d$) target cells as described under *Materials and Methods*. Data are reported as the mean channel fluorescence and represent three separate experiments for each group.

Effects of Low-dose IL-12 (0.1 μg/Day) on Alloantibody Responses

The reduction in sera IgM in WT allograft recipients after rIL-12 (1.0 μg/day) treatment (Table 2) may have been caused by anti-proliferative or toxic effects on B cell function caused by rIL-12 and/or IFN-γ. To test this possibility, WT cardiac allograft recipients were treated once daily with 0.1 μg of rIL-12 and sera alloantibody levels were assessed on day 7 or 8 after transplantation. No alterations in the pathology of allograft rejection was observed in recipients treated with 0.1 μg of rIL-12 compared to 1.0 μg (data not shown). Further, treatment of WT allograft recipients with 0.1 μg of rIL-12 resulted in significantly less IL-12-induced toxicity, serum IFN-γ concentrations in these animals were undetectable by ELISA, and in vivo Th1 sensitization was similar to untreated allograft recipients (data not shown). These results suggested that the 0.1-μg dose of rIL-12 was ineffective in vivo. However, unlike the higher dose of rIL-12 (1.0 μg), treatment of WT cardiac allograft recipients with 0.1 μg of rIL-12 resulted in an increase in sera IgG2a, but not IgG1 at both days 7 and 8 after transplantation (Table 3). Further, IL-12 treatment augmented sera IgM levels at both time points.

TABLE 3. Low-dose rIL-12 augments IgM and IgG2a production in vivo[a]

	Mean channel fluorescence		
	IgM	IgG1	IgG2a
Experiment 1 (day 7)			
Unmodified	11.90	2.06	2.58
IL-12	28.94	2.67	12.79
Experiment 2 (day 8)			
Unmodified	31.54	8.53	16.31
IL-12	46.57	9.72	49.00

[a] Serum was obtained on day 7 or 8 after transplantation from cardiac allograft recipients either left untreated or injected with 0.1 μg of rIL-12 once daily. Isotype-specific anti-BALB/c alloantibody was assessed by flow cytometry using P815 ($H2^d$) target cells as described under *Materials and Methods*. Data are reported as the mean channel fluorescence. Mean channel fluorescence for isotype controls were 1.65 (IgM), 1.67 (IgG1), and 1.57 (IgG2a).

DISCUSSION

Bioactive IL-12 exists as a p70 heterodimer composed of p35 and p40 subunits (*28, 29*). Each subunit of IL-12 interacts with a distinct component of the IL-12R: p40 binds to IL-12Rβ1 and p35 interacts with IL-12Rβ2 (*19, 20*). Both receptor subunits are associated with members of the Janus kinase family (*30*), and therefore may facilitate IL-12-mediated signal transduction. However, most of the attention has been given to IL-12Rβ2, which associates with JAK2 (*30*). For example, recent evidence supports a requirement for IL-12Rβ2 expression in IL-12-induced phosphorylation of Stat4 (*21, 22*). Further, these studies revealed the importance of the β2 subunit of IL-12R by demonstrating that the unresponsiveness of Th2 to IL-12 in both human (*21*) and mouse (*22*) is a result of loss of IL-12Rβ2 expression by these cells. These results indicate that the binding of the p40 subunit of

IL-12 to IL-12Rβ1 is not sufficient to mediate the bioactivity of heterodimer IL-12. However, we have reported that p40 promotes alloantigen-specific CD8+ Th1 development in the absence of heterodimer IL-12 (*15*). This observation suggests that IL-12 p40 mediates its stimulatory effect through IL-12Rβ1 alone, or that IL-12Rβ1 associates with a yet unidentified component of IL-12R on CD8+ T cells. These possibilities have not been tested. Additional data are emerging that support a biologic role of p40 interacting with IL-12Rβ1 (*1*). Specifically, p35 knockout mice, which are capable of producing p40 in levels similar to WT mice (*31*), are less susceptible to infection with *Listeria* and *Cryptococcus neoformas* compared to p40 knockout mice. Hence, one goal of the current study was to assess the role of IL-12Rβ1 in alloimmune responses both in vitro and in vivo.

IL-12 is a potent stimulator of in vitro alloantigen-specific Th1 development, in that the addition of IL-12 to MLC consisting of WT responder splenocytes resulted in a 10-fold or greater increase in IFN-γ production (Table 1; *15*). Exogenous rIL-12 also markedly augments in vitro Th1 development in mice that are deficient in p35, p40 (*15*) or both subunits of IL-12 (JR Piccotti and DK Bishop, unpublished observations), indicating that T cells of these mice are equipped with a functional IL-12R. In contrast, IL-12 did not alter MLC IFN-γ production by splenocytes of IL-12Rβ1$^{-/-}$ mice (Table 1). This result illustrates the requirement of β1 subunit of IL-12R for IL-12-driven Th1 differentiation in vitro. It should be noted that, although IFN-γ production by IL-12Rβ1$^{-/-}$ splenocytes in MLC was reduced compared to WT values (Table 1), this cytokine was readily detectable by ELISA, suggesting that IL-12 is not an absolute requirement for in vitro Th1 responses.

IL-12 is also a key cytokine involved in promoting cell-mediated immune responses in vivo (*1, 2*). However, what role IL-12 plays in transplant rejection remains unclear. It has been reported that IL-12 has a central role in the progression of acute graft-versus-host disease (GVHD) in mice (*6, 32*). In these studies, neutralizing IL-12 with a polyclonal anti-IL-12 antibody results in the amelioration of acute GVHD (*32*) and, conversely, treatment with exogenous IL-12 converts chronic GVHD into exacerbated acute GVHD (*6, 32*). Further, Williamson et al. (*33*) have reported that neutralizing IL-12 during the inductive phase of GVHD results in a Th1 to Th2 shift evidenced by a reduction in IFN-γ and enhancement of IL-5 and IL-10 production by Con A-stimulated splenocytes. In contrast to these findings, neutralizing IL-12 in mouse vascularized cardiac allograft recipients promotes intragraft Th2 cytokine (IL-4 and IL-10) gene expression; however, these grafts are rejected in an accelerated fashion compared to untreated recipients (*16*). Importantly, in vivo Th1 priming is not inhibited by IL-12 neutralization, indicating that Th1 development can occur independent of IL-12 (*16*). This possibility is further supported by the observation that splenocytes of IL-12Rβ1$^{-/-}$ allograft recipients produce similar concentrations of IFN-γ upon restimulation with donor splenocytes compared to WT recipients (Fig. 4). It does not appear that Th1 development in IL-12Rβ1$^{-/-}$ mice is a result of the interaction of endogenous IL-12 with the low-affinity IL-12Rβ2, as treatment of these animals with rIL-12 did not augment in vivo priming of IFN-γ-producing cells (Figs. 3 and 4).

A second hypothesis tested in the current study was treatment of cardiac allograft recipients with IL-12 would accelerate the rejection process as a result of exacerbated Th1-driven immune responses. Administration of exogenous rIL-12 significantly augmented in vivo sensitization of IFN-γ-producing cells in WT cardiac allograft recipients, as evidenced by increased sera IFN-γ (Fig. 3) and enhanced production of IFN-γ by splenocytes after restimulation with donor alloantigens in vitro (Fig. 4). However, this fulminate Th1 response in vivo did not result in anticipated acceleration of graft rejection when compared to untreated control recipients (Fig. 5). It is possible that induction of high systemic levels of IFN-γ results in an inhibition of immune response as a result of IFN-γ's anti-proliferative properties on effector cell development (*34*). However, graft survival was not prolonged after rIL-12 treatment in the current study. This observation questions the overall importance of Th1 responses in this experimental model, and suggests that the magnitude of Th1-driven alloimmune response may not correlate directly to the severity of graft rejection. Indeed, Th2-driven immune responses are emerging as potential effector cells of rejection in both human and experimental transplantation (reviewed in *35*).

Finally, we examined the influence of rIL-12 administration on allospecific B cell function. In an experimental system in which PVG.RT1^{u} congenic rats were immunized with an isolated alloantigen, Gracie et al. (*36*) reported that treatment with murine rIL-12 (1.0 μg/day for 5 days) after alloimmunization augments levels of allospecific IgG2b and IgG2c, while decreasing IgG1. The authors demonstrated that co-administration of neutralizing anti-IFN-γ mAb abrogated this response, indicating that the enhancement of B cell function by IL-12 was dependent on IFN-γ. When adjusted for body weight, this dose of rIL-12 in the rat is comparable to our 0.1-μg dose in the mouse. In the current study, treatment of WT cardiac allograft recipients with 0.1 μg of rIL-12/day for 6 days increased the level of sera IgG2a compared to untreated recipients on day 7 and 8 after transplantation (Table 3). However, administration of 1.0 μg of rIL-12/day reduced allospecific B cell function, indicated by a decrease in sera IgM (Table 2) and absence of isotype switch to IgG2a. These observations suggest a biphasic response to IL-12 treatment in WT mouse cardiac allograft recipients likely dependent on the concentration of IFN-γ.

In summary, this study illustrates that the β1 subunit of mouse IL-12R is critical for IL-12-driven alloimmune responses both in vitro and in vivo, and that IL-12Rβ2 alone does not transduce IL-12 signaling. These observations are supported by recent reports, which have shown that humans deficient in IL-12Rβ1 exhibit severe impairment in their resistance to infections as a result of intracellular pathogens (*37, 38*). The generation of mice deficient in IL-12Rβ2 will provide an important animal model to evaluate whether β1 subunit of IL-12R alone conveys IL-12 responsiveness in vivo. Specifically, these mice would be useful in determining the mechanism by which p40 subunit of IL-12 enhances CD8+ Th1 development (*15, 16*). Finally, this study questions the importance of Th1-driven alloimmune responses in cardiac allograft rejection, as exacerbated Th1 responses induced by IL-12 failed to accelerate graft rejection in this model.

Acknowledgments. The authors thank Drs. Jeanne Magram and Maurice K. Gately for generously providing IL-12Rβ1$^{-/-}$ mice and murine rIL-12, and for their helpful discussions. The authors also thank Brooke Schuemann for technical support.

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Received 18 August 1998.
Accepted 4 December 1998.

0163-4984/01/7901-0015 $12.00

APPENDIX 4

Zinc Inhibits the Mixed Lymphocyte Culture

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Received June 13, 2000; Accepted July 10, 2000

ABSTRACT

The mixed lymphocyte culture (MLC) is an established clinical method for bone marrow transplantation, as it serves as an in vitro model for allogenic reaction and transplantation. We previously showed that cytokine release into the supernatant is a more specific and sensitive parameter for cross-reactivity in the MLC than the common measurement of cell proliferation. Therefore we tried to find an inhibitor of the MLC in vitro with the least side effects in vivo, measuring interferon (IFN)-γ as one of the most important cytokines in posttransplant medicine. Earlier studies showed that zinc is an important trace element for immune function with both stimulatory and inhibitory effects on immune cells. We found that slightly elevated zinc concentrations (three to four times the physiological level), which do not decrease T-cell proliferation in vitro nor produce immunosuppressive effects in vivo, suppress alloreactivity in the mixed lymphocyte culture. In this report we analyzed the mechanism whereby zinc influences the MLC to possibly find a nontoxic way of immunosuppression.

Index Entries: Mixed lymphocyte culture (MLC); mixed lymphocyte reaction (MLR); trace elements; zinc.

INTRODUCTION

The mixed lymphocyte culture (MLC) is a well-established and important tool for determination of compatibility between host and donor

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in transplantation medicine, as it serves as an in vitro model for allogenic reaction *(1,2)*. It is common to measure T-cell proliferation in the MLC, but it was recently shown that cytokines are more specific and sensitive parameters for the prediction of a possible graft rejection, as they play a critical role in the posttransplant response *(3–5)*. The TH1-cytokine interferon-γ (IFN-γ) was identified as the most important factor within the cytokine cascade in the MLC *(6)*. It is known to induce cytotoxic T-lymphocytes (CTL) *(7)* by enhancing the expression of both major histocompatibility complex (MHC) class I and MHC class II molecules *(8)*. The IFN-γ response mainly depends on HLA-DR differences and it therefore well represents reactivity between two individuals in the MLC *(9)*.

In transplantation medicine, cyclosporin A, FK506, and other substances are used to prevent graft rejection. In vitro experiments revealed an inhibition of the MLC *(10)*, but, unfortunately, all of these immunosuppressants show a wide range of toxicities in vivo, such as nephrotoxicity, neurotoxicity, and, probably, carcinogenicity *(11–13)*. As we are beginning to understand the molecular mechanisms of cyclosporin A and FK506 function better and better, one of the major aims is to find similar substances with less toxicity.

Zinc within the physiological range (12–16 μ*M*) is an important trace element for immune function *(14)*. Zinc deficiency in vivo could be linked to various clinical symptoms such as impaired immune response with regard to decrease in number, differentiation, and function of T-lymphocytes and natural killer (NK) cells as well as decreased activation of monocytes and phagocytosis by macrophages, resulting in a high incidence of bacterial, viral and fungal infections. These symptoms, in the most severe form shown in the hereditary disease acrodermatitis enteropathica caused by malabsorption of zinc, are completely reversible after adequate substitution of zinc *(15)*. On the other hand, high concentrations of zinc (about eight times the physiological level) led to cytotoxic effects with impairment of all T-cell functions, and inhibition of monokine induction by superantigens such as zinc is also important for the binding of some bacterial superantigens to the β-chain of the MHC class II molecule *(16,17)*. Optimal immune-cell function hence requires a well-balanced zinc level.

In the following study, we investigated whether zinc is able to impair alloreactivity in the MLC at concentrations with neither cytotoxic effects in vitro nor toxic side effects in vivo.

MATERIALS AND METHODS

Preparation of Lymphocyte Cultures

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by density centrifugation over Ficoll–Hypaque (Biochrom, Berlin, Germany), washed twice with phosphate-buffered saline (PBS, Gibco, Berlin, Germany) and resuspended in RPMI-1640 medium (Biochrom) supplemented with 10% heat-inactivated fetal calf

serum (FCS, low endotoxin, myoclone quality; Life Technologies, Eggenstein, Germany), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all obtained from Biochrom, Berlin, Germany). The cells were adjusted to a final concentration of 2×10^6 cells/mL. Equal volumes of cell suspensions of two donors were seeded in samples to a final volume of 1 mL into pyrogen-free 24-well culture plates (Falcon, Heidelberg, Germany). For controls, 1 mL of the adjusted cell suspension was cultured separately. The cultures were incubated for 5 d at 37°C in a 5% humidified CO_2 atmosphere after addition of the appropriate amount of zinc.

Zinc Preparations

Zinc sulfate (Sigma, Deisenhofen, Germany) was dissolved in sterile water to achieve a zinc stock solution of 10 mM. This solution was further diluted in unsupplemented protein-free medium (PFM, Ultradoma, BioWhittaker) at a ratio of 1 to 2 and then sterile filtered. To achieve the final concentrations, PFM was used. The zinc solution was added to the cultures in a volume of 10% of the final culture volume.

Determination of Cytokines

The culture supernatants were harvested after 5 d and stored at –80°C. The quantification of the cytokine release into the supernatant was performed by enzyme-linked immunosorbent assay (ELISA) technique (for IFN-γ provided by Bender Med Systems, Vienna, Austria). Results were measured in picograms per milliliter at 450 nm using an ELISA plate reader (Anthos Labtec, Salzburg, Austria).

Flow Cytometry

Propidium iodide (PI) staining was performed by using a stock solution of 1 mg/mL (PI, Sigma). Cells (1×10^6/mL) were incubated with 10 µL of PI stock solution for 20–30 min to allow intercalation of PI in double-stranded DNA. Finally, PI staining was measured at a wavelength of 620 nm in a flow cytometer (Coulter, Krefeld, Germany).

Statistical Analysis

The results are expressed as median values. The significance is taken by Student's *t*-test analysis.

RESULTS

Influence of Zinc on Mixed Lymphocyte Cultures

We harvested the supernatants of zinc-supplemented mixed lymphocyte cultures (MLC) on d 5, proven to be the maximum of the IFN-γ secretion *(18)*. Analyzing IFN-γ release in 20 MLC experiments supplemented with different concentrations of zinc, we found expected amounts of IFN-

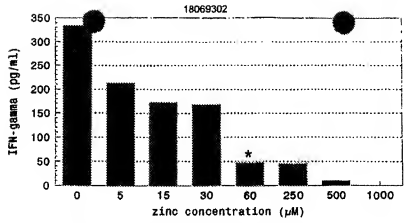

Fig. 1. IFN-γ secretion in the MLC after zinc supplementation. Zinc concentrations up to 1 m*M* were added to mixed lymphocyte cultures; controls remained unsupplemented. Secretion of IFN-γ in the culture supernatants was determined by ELISA after 5 d of culture. Median values of $n = 20$ experiments are expressed in picograms per milliliter. Significance was calculated by the Student's *t*-test (*$p = 0.0017$).

γ (334 pg/mL) in the supernatant of control MLC without zinc addition, whereas increasing zinc concentrations led to a dose-dependent reduction of the IFN-γ level. At 60 μ*M*, the IFN-γ production was significantly diminished (48 pg/mL, $p = 0.0017$); at 500 μ*M*, no IFN-γ was detectable (Fig. 1).

In order to prove our hypothesis that this result—that zinc concentrations of 60 μ*M* inhibit the MLC—was the result of a specific effect in the MLC and not to a loss of T-cell vitality, we added zinc concentrations of up to 5 m*M* to PBMC and measured cell viability by flow cytometry after an incubation time of 48 h. Figure 2 shows that 93.2% of the cells are still vital after addition of 50 μ*M* zinc and 92.3% with medium supplementation of 100 μ*M* zinc compared to controls without zinc addition with 91.3% viability. Zinc concentrations as high as 250 μ*M* causes a reduction of cell survival of 33% (Fig. 2).

For further analysis of possible mechanisms responsible for this inhibition, we preincubated PBMC with 50 μ*M* zinc for 20 min and then cocultured these two populations in the MLC. The results reveal a marked influence of the point of time at which zinc is added to the culture: Preincubation of PBMC led to a greater reduction of IFN-γ than simultaneous zinc supplementation to the MLC (Fig. 3).

DISCUSSION

The human mixed lymphocyte culture (MLC) is an important method to test donor–recipient compatibilty in bone marrow transplantation. It could be shown that cytokine release, especially IFN-γ, has a very good predictive value with regard to the transplantation outcome (*3*), as cytokines play a major role in the generation of an alloreactive immune response and

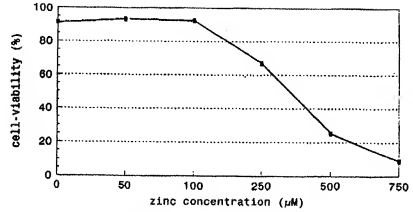

Fig. 2. Viability of PBMC after zinc supplementation. Zinc concentrations of up to 750 µM were added to unstimulated PBMC; controls remained unsupplemented. Cell viability was determined by flow cytometry after an incubation time of 48 h. One representative experiment is shown, values are expressed in percent of the total cell population.

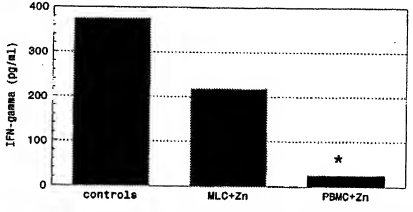

Fig. 3. Effect of preincubation of PBMC with zinc. Zinc in a concentration of 50 µM was added to the MLC simultaneously (MLC + Zn); PBMC was preincubated with 50 µM zinc for 20 min and then cocultured in a MLC (PBMC + Zn); controls remained unsupplemented. Medians of $n = 10$ experiments are presented. Significance was calculated by the Student's t-test (*$p = 0.01$).

for the induction of graft rejection in vivo *(4,5)*. Taking this in vitro model, it has always been the aim to inhibit proliferation of immune cells in order to find a way to prevent graft rejection in transplantation medicine. Landolfo et al. inhibited T-cell reactivity by the addition of anti-IFN-γ both in vitro and in vivo *(19)*; others showed a reduced graft rejection rate by repeated injections of monoclonal anti-IFN-γ antibodies in a skin-, heart-, or pancreas-tissue transplantation situation *(20–22)*.

In vivo substances like cyclosporin A or FK506 are broadly applied, as they are capable of prolonging graft survival. In vitro, they show an inhibitory effect on T-cell proliferation in the MLC *(10)*. Yet, all of these therapeutical agents cause major side effects (e.g., nephrotoxicity, neurotoxicity, and others), which lead to a limitation of their use *(11–13)*.

Zinc is an essential trace element with great influence on immune function. The physiological plasma level of zinc ranges from 12 to16 μM. In our study, we applied zinc concentrations up to 100 μM, which can be reached by pharmacological application of zinc in vivo without causing side effects *(23)*.

We found that zinc concentrations of 60 μM, four times the physiological level, inhibit alloreactivity in the MLC. It is unlikely that the reduction of IFN-γ is the result of a loss of T-cell activity, as it could be shown earlier that T-cells are still able to proliferate in medium supplemented with zinc concentrations as high as 100 μM *(24)*. Furthermore, we analyzed the viability of the PBMC by flow cytometry, showing that a concentration of 250 μM is required to reduce cell viability by 33% (Fig. 2).

Increased zinc levels of over 100 μM cause unstimulated human PBMC to release cytokines *(25)*. This stimulatory effect of zinc is only seen in the presence of accessory cells, especially monocytes, as mostly IL-1 proved to be an essential cosignal for T-cell activation by zinc. Higher concentrations of zinc impair all T-cell and monocyte function by inhibition of the IL-1 receptor type I-associated protein kinase (IRAK), thus blocking the intracellular-signal transduction pathway at a very early stage *(24)*.

In our study, we applied zinc in concentrations that neither show cytotoxic effects nor reach stimulatory level. Therefore, there seems to be a specific effect of zinc on the responding T-cells in the MLC.

The results of earlier studies proposed an oligoclonal pattern of T-cell stimulation in the MLC similar to T-cell activation by superantigens *(3)*. Furthermore, a highly altered Vβ repertoire of T-cells infiltrating long-term rejected kidney allografts were discribed *(26)*. Superantigens bind directly and partially with high affinity to major histocompatibility complex (MHC)-class II proteins, especially to HLA-DR. T-Cell activation is achieved by the formation of a complex of the Vβ-chain of the T-cell receptor (TCR), the MHC molecule, and the superantigen. This binding is regulated by zinc, as zinc itself does not interact with the MHC molecule directly *(27)*. We previously showed that the HLA-DR and HLA-DQ-molecules have the greatest influence on cytokine release in the MLC and thus on the outcome of a transplantation in vivo *(9)*.

There are two main possible explanations for the phenomenon described. First, zinc in the applied concentration could saturate the MHC and, therefore, prevent a binding between TCR and MHC. In order to prove this hypothesis, we preincubated PBMC with zinc and then cocultured these populations. If an extracellular mechanism were actually responsible for the inhibition of the MLC, we would expect no significant difference in IFN-γ secretion in either setting. Preincubation of PBMC

resulted in a markedly lower IFN-γ secretion than the culture of two PBMC populations with simultaneous zinc supplementation to the MLC (Fig. 3), so that it seems more likely that zinc interferes with the intracellular signal transduction in the MLC. Therefore, zinc may regulate the alloreactivity of T-cells and might be an explanation for increased preterm delivery and abortion in zinc-deficient pregnant women (*28,29*). As mentioned earlier, higher concentrations of zinc are able to block the intracellular signal transduction pathway by inhibition of IRAK. We propose that the stimulation of T-cells by an HLA-different cell population can be blocked by zinc via specific inhibition of phosphorylation processes, leading to a diminished signal transduction in the cell. This results, among other things, in reduced secretion of cytokines, which should lead to less graft rejection in vivo. Various protein kinases such as cAMP- and cGMP-dependent protein kinases as well as protein tyrosine kinases are involved in zinc-induced cell stimulation and zinc also influences gene expression of different immunologically relevant transcription factors such as nuclear factor (NF)-κB and metallothionein transcription factor (MTF-1) as well as others. Which alteration of signal transduction zinc exactly inhibits the MLC remains the subject of further investigation. Because the MLC is inhibited by very low zinc concentrations, this inhibitory effect seems to be a specific pathway.

In conclusion, zinc could become an immunosuppressant in transplantation medicine without toxic side effects, which still leaves the immune system with the ability for phagocytosis. The infection rate will therefore be reduced compared to current immunosuppression. However, this has yet to be proven in in vivo transplantation models.

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